THE INTERACTION OF ELECTRIC FIELDS WITH
VESICLES AND CELLS: A STUDY ON
ELECTRODEFORMATION AND
ELECTROPORATION

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ABSTRACT OF THE DISSERTATION

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The application of electric pulses to cells or vesicles induces complex responses. When electric pulses are applied, a lipid membrane may become porated, which is a phenomenon known as electroporation. The lipid membrane may also deform under electromechanical and electrohydrodynamic forces applied via electric fields, which is a phenomenon known as electrodeformation. Electroporation is widely employed in both biological research and clinical applications where access to the cytoplasm is desired. Electrodeformation, on the other hand, can be harnessed as a means to probe the membrane’s properties, and to detect pathological changes in cells. Despite its broad applicability, electroporation still suffers from low delivery efficiency and cell viability, in part due to a lack of a fundamental understanding of the mechanisms involved in this technique. Meanwhile, the electrodeformation phenomenon only received attention in the past decade. A significant body of data only became available in the recent years due to the development of high-performance optical imaging systems which captured the fast dynamics associated with electrodeformation. In this work, we designed and implemented experiments to study the electrodeformation of vesicles, and to investigate the transport mechanisms during electroporation. First, we characterized the prolate
deformations of giant unilamellar vesicles under strong DC electric fields. In some cases, the aspect ratio of a deformed vesicle exceeded 10, representing a strong-deformation regime never explored before. Second, we studied the spatial and temporal transport of molecules into single cells with fluorescence microscopy. The results demonstrated that electrophoresis is the dominant mode of delivery during electroporation. Furthermore, we found that field-amplified sample stacking (FASS), an electrokinetic mechanism, mediated an inverse correlation between delivery and the extracellular buffer electrical conductivity. Third, we employed a two-stage electroporation pulsing scheme to permeabilize the membrane and to electrokinetically-mediate species transport into the cytoplasm. We found that the delivery and viability can be both maximized by optimizing the parameters of the pulses. This dissertation contributes to the electrodeformation and electroporation fields by generating insights into deformation and transport mechanisms based on physical principles. More importantly, the developed experimental tools and optimized protocols may improve the efficacy of both methods.
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Chapter 1
Introduction

1.1 General Introduction

Cell based therapies following injury deliver agents to provide trophic support and protect against secondary insults, as well as to deliver cells themselves to restore lost populations of cells [3, 14, 42, 43, 44]. This option of regenerative medicine is a fast developing field that can revolutionize the treatment of human disease through the development of innovative new treatments that offer a faster, more effective recovery with substantially fewer repercussions or risk of complications to improve healthcare overall [14, 21, 42]. Cellular therapies involve the manipulation of stem cells or patient cells, and their subsequent transplantation into the patient [8, 9, 77, 126]. Despite extensive research, cell manipulation *in vitro*, involving either stem cells or progenitor cells, remains a difficult matter that requires a strictly regulated cellular response and carefully controlled cellular environment [8, 9, 14, 42, 44, 77, 126]. The root cause for these shortcomings is the heterogeneous behavior of cells and the complexity of multicellular systems that require rigorous and laborious single cell resolution studies [8, 42, 77, 87, 99]. Moreover, existing cell manipulation techniques either require the use of bulk techniques that lack single-cell accuracy, or manual methods that are able to provide single-cell accuracy but at significantly lower throughputs and repeatability [87, 99]. Developing current and new cell manipulation methods not only provides a means for controlling cellular properties and functionality, but also further the understanding of the complex cellular mechanisms necessary to engineer homogeneous cell populations *in vitro* for clinical treatments, tissue engineering, and research-based pharmacology and toxicology studies.

In the past three decades, various physical force field-based techniques have been
exploited extensively to trap, and manipulate biological cells physically and genetically [28, 65, 74, 113]. In particular, electric-field based techniques have emerged as powerful tools in gene and drug delivery, membrane-fusion, cell movement guidance, and measurement of physical and dielectric properties of cells [20, 46, 65, 74]. With electric field based techniques, single and large number (millions) of cells could be manipulated, and the protocols are usually straightforward requiring relatively simpler equipment when compared to other manipulation methods [65].

Herein, we focus on two electric-field based techniques for cellular manipulation namely, electroporation and electrodeformation. In electroporation, an electric field is employed to create pores in the cellular membrane, and drive molecules into/out of the cytoplasm. Electroporation has been widely utilized in areas such as drug and gene delivery, protein insertion, and cancer therapy, among others [1, 13, 20, 106]. In addition to electroporation, electromechanical and electrohydrodynamic forces applied to cells causes the membrane and cytoskeleton to deform, a phenomenon referred to as electrodeformation [4, 22, 89, 90, 125]. This technique has been harnessed as a means to probe the viscoelastic properties of cells [25, 60, 70, 125], to detect the pathological changes in cells [119], and to mediate differentiation of stem cells [104]. Although both methods are currently being applied, each suffers independently as they have not been optimized, and there are still many unanswered questions and discrepancies limiting the improvement of both techniques [1, 20, 22, 66, 107, 116, 125].

This thesis presents a thorough study on a new regime of membrane electrodeformation and novel approaches to enhance delivery and cellular viability associated with electroporation. First, we systematically characterize the electrodeformation of giant lipid vesicles under the combination of strong DC electric fields and high intra-to-extra-vesicular conductivity ratios with direct optical observation. Second, we investigate the transport mechanism, and identify a critical electrokinetic mechanism associated with electroporation to improve molecular uptake into cells. The acquired knowledge is utilized to conduct a parametric study to gain advantage of the electrokinetic transport mechanisms during electroporation.
1.2 Electrodeformation of Vesicles

Electrodeformation refers to the deformation of cell or vesicle lipid membranes under the application of electric field. When subjected to applied electric pulses, membranes deform under electromechanical and electrohydrodynamic forces resulting from the charge accumulation at the vesicle poles. In fundamental membrane research, electrodeformation has been used as a tool to probe the mechanical properties of lipid membranes such as the bending rigidity [59, 75] and the elastic modulus of stretching [60]. For cellular based research, electrodeformation can be leveraged to study the changes of cellular elasticity to probe pathological changes such as spherocytosis and metastatic competence of eukaryotic cells [119]. Although research in this area is not recent [49, 50, 59, 70, 118], a significant body of data only became available in the recent years with the development of high-performance optical imaging systems [4, 22, 54, 89, 90, 112].

1.2.1 Vesicles as Spherical Membrane Models

Biological membranes are an integral part to the structure and function of all cells. Membranes are impermeable and essential to maintain the integrity of the intercellular lumen and organelles. The membrane regulates transport into and out of the cell and responds to external and internal stimuli. According to the fluid mosaic model suggested by Singer and Nicolson, shown in Fig. 1.1, cellular membranes are fluid-like bilayers comprised of lipids embedded with proteins [97]. The three classes of amphipathic lipids, phospholipids, glycolipids and sterols, contain a hydrophillic part (glycerol and headgroup moieties) and a hydrophobic part (fatty acyl chains). The amphipathicity of membrane lipids makes them unstable when placed in water, and gives them the ability to spontaneously form closed spherical compartments. Moreover, each type of lipid molecule has a defined geometry which influences the physical properties of the membrane such as curvature and thickness. The composition of lipid membranes varies based on the function, state, and chemical composition of the lumen of each compartment.

Due to the functional complexity of biological membranes, the scope of this unit will
Figure 1.1: Membrane mosaic model suggested by Singer and Nicholson [97]. The membrane is comprised of a lipid bilayer embedded with proteins.

be limited to the chemistry of the membrane structure and the transport of substances across membranes. Specifically, vesicles have been extensively used to study the stability of membranes, interactions between bilayers, and the movement of solutes across membranes. Vesicles are spherical lipid bilayers that can be artificially prepared. Three types of vesicles can be formed experimentally as shown in Fig. 1.2: the multilamellar vesicles (MLVs), the small unilamellar vesicles (SUVs), and the giant unilamellar vesicles (GUVs). The MLVs are vesicles with micron range diameters where several smaller vesicles are contained within one larger vesicle. This type of vesicle does not correctly model the plasma membrane due to its composition of numerous bilayers. The SUVs are vesicles with diameters between 20 nm and 200 nm, and they are used as chemical delivery shuttles for DNA and other biologically active molecules. Furthermore, SUVs may also be applied to help in the understanding of the curvature dynamics of vesicles due to their nanometer size. Finally, the GUVs are systems with comparable diameters as biological cells, between 10-100 µm, hence they can be directly observed with a microscope. More importantly, their composition as well as their internal and external environment can be controlled. The GUVs have been adapted as the most
Figure 1.2: The different spherical lipid membranes used in studying cellular membranes. (a) Multilayer vesicles (MLVs). (b) Small unilamellar vesicles (SUVs). (c) Giant unilamellar vesicles (GUVs).

A basic fundamental model for studying the mechanical properties of cellular membranes.

1.2.2 Transmembrane Potential and Electrotension Force

The membrane have a relatively lower dielectric constant ($\epsilon_m = 2$) and is much less conductive (specific membrane conductance about $\sigma_m = 1 \text{ ms/cm}^2$) than the suspending medium and the cytoplasm. Therefore, under the influence of an applied electric field, free ions in the intra-and-extracellular environments accumulate at the interface. This charge build-up at the membrane causes a change in the transmembrane potential. Assuming the membrane is a thin bilayer ($h \sim 5 \text{ nm}$), and behaving as a capacitor, the field-induced transmembrane potential can be calculated by [57]:

$$ V_m = \frac{3}{2} E a \cos \theta (1 - e^{t/\tau_{\text{charge}}}) $$

(1.1)

where $E$ is the applied external electric field, $a$ is the radius of a cell, $\theta$ is the angle between the direction of the electric field and the cell surface normal, and $\tau_{\text{charge}}$ represents the membrane charging time, given by:

$$ \tau_{\text{charge}} = a C_m \left( \frac{1}{\sigma_i} + \frac{1}{2\sigma_e} \right) $$

(1.2)
where $C_m$ is the membrane capacitance, and $\sigma_i$ and $\sigma_e$ are the intra-and-extracellular medium conductivities, respectively. Moreover, the Maxwell stresses generated from the interaction between the membrane molecules and the applied electric field induces a perpendicular tension at the cell or vesicle poles. This tension is given by [70]:

$$\sigma_{el} = \epsilon \epsilon_0 \left( \frac{h}{2h_e^2} \right) V_m^2$$

(1.3)

where $\epsilon$ is the dielectric constant of the aqueous solution, $\epsilon_0$ is the vacuum permittivity, $h$ is the total bilayer thickness, and $h_e$ is the dielectric thickness. This electrotension force is the root cause for electrodeformation.

### 1.2.3 Electrodeformation of Vesicles During Applied Electric Fields

Electrodeformation may occur under both AC and DC electric fields (Fig. 1.3). When investigating this phenomenon, most authors use vesicles as a model system (instead of biological cells) due to their relative simplicity and controllability. An AC field in general induces a variety of relatively stationary deformations based on the frequency domain and conductivity ratio as shown in Fig. 1.3(b). For this reason, it is used to systematically quantify membrane responses, often in the absence of membrane poration. Helfrich and co-authors, for instance, analyzed area changes of deformed vesicles to infer the bending rigidity of the membrane [59, 75]. Dimova and co-authors performed extensive studies to characterize vesicle morphological types (prolate, oblate, and spherical) in the parametric space of field frequency and intra-to-extra-vesicular conductivity ratio [89, 90]. These studies were later complemented with an electrohydrodynamic theory by Vlahovska et al. to interpret the observed trends and regimes [112]. Electrodeformation under DC fields (Fig. 1.3(a)), on the other hand, is transient and dynamic. Due to the high field strength normally used in DC studies, membrane poration often occurs concurrently. In a series of work, Neumann and co-authors studied the electroporative deformation of vesicles hundreds of nanometers in size [53]. At this range, direct optical observation is difficult, and alternative techniques such as conductometrical and turbidimetrical measurements were employed. The data were analyzed
to extract useful information such as pore statistics, vesicle volumetric reduction, and the correlation between membrane curvature and pore formation. In contrast, direct observation of giant vesicles (typically a few tens of microns in diameter) under the action of a field can reveal rich and complex details of the dynamic process. Riske and Dimova developed a high-speed imaging system to investigate the deformation-relaxation of vesicles under DC electric fields [89]. The results were discussed in relation with the electrotension (the Maxwell stress) on the membrane, as well as various time scales (the viscous relaxation and the pore relaxation), which are important in understanding the membrane relaxation mechanisms post-pulsation. In a companion work, the same authors observed transient, cylindrical deformations when the vesicles were suspended in a salt solution [90].

In this work, the electrodeformation of giant unilamellar vesicles under strong DC electric fields was investigated. Specifically, the degree of deformation was quantified as a function of the applied field strength, and the electrical conductivity ratio of the fluids inside and outside of the vesicles. The vesicles were made from L-\(\alpha\)-phosphatidylcholine with diameters ranging from 14 to 30 \(\mu\)m. Experiments were performed with field strengths ranging from 0.9 to 2.0 kV/cm, and intra-extra-vesicular conductivity ratios varying between 1.92 and 53.0. With these parametric configurations, the vesicles exhibited prolate elongations along the direction of the electric field. The degree of
deformation was in general significant. In some cases, the aspect ratio of a deformed vesicle exceeded 10, representing a strong-deformation regime previously not explored. The aspect ratio scaled quadratically with the field strength, and increased asymptotically to a maximum value at high conductivity ratios. Appreciable area and volumetric changes were observed both during and after pulsation, indicating the concurrence of electroporation. With the help of my colleague Dr. Jianbo Li, a theoretical model was developed to predict these large deformations in the strongly-permeabilized limit, and the results were compared with the experimental data. Both agreements and discrepancies were found, and the model limitations and possible extensions are discussed. This work has been summarized in a publication in Physical Review E journal [93].

1.3 Electroporation-Mediated Molecular Delivery

In the past three decades, electroporation-mediated molecular delivery has attracted significant attention due to its great potential for cell manipulation in biological research and medical applications [1, 15, 20, 36, 66, 67, 73, 91]. In this technique, an applied electric field transiently increases the membrane conductance, and the lipid membrane becomes permeable. The degree of permeabilization can be either irreversible (IRE) or reversible depending on the electric field parameters. When the optimum field parameters are surpassed, the cellular membrane disintegrates and DNA is damaged leading to irreversible permeabilization which induces either necrotic or apoptotic cell death. Due to its potent detrimental effects on cells, IRE has been shown to be effective in applications where cell death is desired such as biofouling control, debacterialization, and ablation of solid tumors as a drug-free cancer therapy [69, 76, 92, 96]. On the contrary, in reversible permeabilization the membrane is transiently disrupted so that impermeant molecules such as DNA, RNA, or drug molecules, can enter the cytosol and the nucleus, while maintaining cell functionality and viability [36, 66, 107]. The latter is the focus of the current work.

Electroporation-mediated molecular delivery is preferred in some applications over other delivery methods. The electroporation process involves only a physical interaction between the electric field and the cellular membrane [38, 45, 73, 74]. Furthermore, the
electroporation process is capable of transporting molecules with a wide range of sizes and is less dependent on cell type. As opposed to competitive methods such as chemical transfection and viral infection, electroporation is cost efficient, easy to operate, and has lower toxicity effects [66, 67, 88]. Due to these advantages electroporation has been applied in a wide variety of *in vitro* and *in vivo* applications. For instance, in various clinical studies, electroporation has been harnessed for DNA vaccination, and has shown great potential for the treatment of a variety of cancers including skin, lung, and breast tumors, bone metastases and leukemia, among others [20, 24, 29, 46, 61, 100, 124].

Despite its great potential and extensive applications, electroporation still suffers from significant limitations, including low delivery efficiency and low cell viability. Although improvements have been sought for specialized cases, the approaches were by and large empirical and *ad hoc*, and could not be generalized. As a result, the delivery efficiency is not reliable, and may vary by several orders of magnitude under different experimental conditions [107, 120]. The main bottle neck in overcoming these limitations is a lack of fundamental understanding of the physical mechanisms involved in electroporation and the lack of accurate prediction tools.

1.3.1 Permeabilization and Transport Mechanisms

Despite extensive research, molecular delivery by electroporation remains challenging and complex in nature [107, 116]. Molecular delivery by electroporation is an intricate physical process involving two major aspects: (I) permeabilization of the membrane [17, 58, 71, 74, 107], and (II) transport of species into the permeabilized cell [63, 72, 86, 103]. The applied electric pulse introduces an electric potential drop across the cell membrane, which is termed the transmembrane potential (TMP) [17, 18, 40, 47, 57, 58, 71]. When the TMP exceeds a critical threshold, aqueous, conducting pores/defects begin to form on the membrane [12, 105, 108]. Subsequently, the membrane conductance is significantly increased and molecular transport into and out of the cell is allowed.

Research conducted over the past three decades has led to a relatively mature understanding of membrane permeabilization and well-developed theoretical models that describe this phenomenon [58, 71, 117]. The permeabilized area has two distinct pore
populations [58, 91, 98, 123]: small pores, which allow for the transport of molecules smaller than 4 kDa, and can remain open from several seconds to minutes after pulsation [37, 98, 123], and large pores, which allow for the transport of molecules larger than 4 kDa, and close within one second after the electric field is switched off [37, 72, 91, 98]. The transmembrane dynamics are well captured by the Smoluchowski equation (SE) [5, 31, 78, 111], which statistically describes the evolution of the pore population as a function of the driving TMP. Several groups adopted the SE equation to build models that predict the membrane permeabilization and directly track the evolution of pore size and population distribution [39, 52, 58, 98]. The theoretical framework in these studies not only provides a comprehensive understanding of membrane permeabilization, but also offers quantitative prediction tools to correlate with experimental data, and to help develop both existing and emerging applications of electroporation.

Although much is already known concerning the permeabilization of the cellular membrane, considerably less is known about the transport phase of electroporation [98, 103, 107]. Even though several transport mechanisms have been identified to contribute to delivery, none were able to provide a full explanation of all the experimental observations [1, 72, 86, 91, 98, 103, 107, 114]. For instance, in DNA electrotransfer across the membrane, electrophoresis and membrane-DNA interactions, such as endocytosis, are involved [1, 26, 73, 91, 98, 103, 107, 114]. Similarly, for smaller molecules such as propidium iodide (PI), calcium ions, and most drug molecules, which have molecular weights less than 4 kDa, simple diffusion and electrophoresis may influence the delivery [33, 35, 63, 68, 79, 86, 91]. Although each of these mechanisms can contribute to delivery, different findings exist on their relative importance [1, 10, 23, 33, 35, 68, 86, 91, 103]. For example, phototube measurements by Pucihar et al. revealed that free diffusion of PI post-pulsation contributed to most of the collected fluorescence signal, whereas a series of transdermal drug delivery experiments suggested that electrophoresis of charged molecules had the major contribution [19, 82, 83, 86, 109, 110].

Herein we first investigate the transport kinetics during electroporation by varying the conductivity of the extracellular buffer and identify key mechanisms. The molecular delivery into cells is quantified and compared directly with modeling predictions. Then,
we exploit the transport mechanism by separating the applied electric field during electroporation into two distinct phases - a ‘permeabilizing’ phase and an ‘electrophoretic transport’ phase – in order to maximize both molecular delivery and cell viability.

1.3.2 Electrokinetic Transport During Electroporation

A careful literature review of electroporation research and consideration of electrohydrodynamic theory reveals that the transport and accumulation of molecular species associated with electroporation cannot be simply due to passive diffusion, but rather is mediated by both electrokinetic parameters as well as specific geometry-driven electrokinetic phenomena [62, 63, 64]. For instance, during electroporation-mediated transport, the net result of electrophoretic mobility of charged species increased the molecular uptake into cells several orders of magnitude above the amount predicted by diffusion of charged particles [63, 64]. Furthermore, not only did molecules experience significant transport following electroporation, but they also accumulated within the cell [34, 35, 63, 107]. This observation cannot be explained solely by the increase in transport rates due to electrophoretic movement. A careful consideration of the system, particularly the electrical geometry of the cell, strongly suggests that a specialized electrokinetic mechanism – Field amplified sample stacking (FASS) – contributes significantly to the observed transport, as described below.

FASS is an electrokinetic technique commonly used in the development of electrophoretic assays in micrototal analysis systems. This mechanism relies on differences in electrophoretic velocities induced by buffers with different electrical conductivities [7, 121]. A schematic of FASS is shown in Fig. 1.4(a). Initially, a buffer with high electrical conductivity (σ2) is placed next to one with lower conductivity (σ1); the sample analytes are initially dispersed in the low conductivity zone (Fig. 1.4(a), top). When an electric field is applied (from left to right), the resulting electric field is higher in the low conductivity zone (E1 > E2, Fig. 1.4(a), middle) because the overall electric current must be conserved, according to the ohmic law of electric current conservation: \( \sigma_1 E_1 = \sigma_2 E_2 = J \), with \( J \) being the Ohmic current density. When the positively
charged analyte ions electromigrate towards the right, because their electrophoretic velocities are linearly proportional to the local electric field, they experience a sudden “slow-down” at the conductivity interface and “stack” (Fig. 1.4(a), bottom).

The electrical configuration of a typical electroporation experiment has great resemblance to that used in FASS. An idealized model of a single cell (Fig. 1.4(b)) comprises a cytoplasm of conductivity $\sim 5 \text{ mS/cm}$, and a thin membrane with conductivity, $\sim 0.1 \text{ mS/cm}$ [30, 79]. The conductivity of the suspension buffer is typically a few times lower than that of the cytoplasm [35]. When an electric field is applied, the membrane becomes permeable to both ionic currents and molecular transport (Fig. 1.4(b) - the dashed boundary). Because the electrical conductivity is higher within the cell, the resulting electric field is lower than outside the cell. The observed similarity in the conductivity and electric field distribution of FASS and in electroporation leads to the hypothesis that molecular delivery also behaves like sample stacking.

In the current work, we conducted a series of experiments to demonstrate the importance of electrokinetic transport during electroporation, which will also identify rational, theory-based means of improving transport efficiency and cell viability in vitro. Therefore, the transport mechanisms in electroporation-mediated molecular delivery are experimentally investigated and quantified. In particular, the uptake of propidium iodide (PI) into single 3T3 fibroblasts is investigated with time- and space-resolved fluorescence microscopy, and as a function of extracellular buffer conductivity. During the
pulse, both the peak fluorescence intensity and the total integrated fluorescence intensity exhibited an inverse correlation with extracellular conductivity. This behavior is mediated by an electrokinetic phenomenon known as Field-Amplified Sample Stacking (FASS). Furthermore, the respective contributions from electrophoresis and diffusion have been quantified; the former is shown to be consistently higher than the latter for the experimental conditions considered. The results are compared with a compact model to predict electrophoresis-mediated transport, and a good agreement is found between the two. The combination of the experimental and modeling efforts provide an effective means for the quantitative diagnosis of electroporation. This work has been summarized in a publication in *Biochimica et Biophysica Acta (BBA) - Biomembranes* [94].

### 1.3.3 Delivery and Viability Enhancement

In typical electroporation, a single pulse is delivered to form pores in the cell membrane and to drive species into or out of the cell. However, the field strength necessary for permeabilization is significantly greater than that required for effective transport of ions and macromolecules, and can therefore be detrimental to the viability of the cells [32, 48, 80]. For instance, a long pulse duration at high field strengths necessary for electroporation can significantly damage cells, but the same duration at low field strengths may enhance delivery by increasing transport time [11, 32, 84, 103, 123]. Although improvements of transfer efficiency and cell survival rate have been attempted in laboratory research, the rationale behind these efforts has been by and large based on qualitative speculation, not on established physical principles [101, 123]. Unfortunately, the current protocols are *ad hoc* and empirical in nature, and a general methodology based on solid physical principals for the optimization of electroporation performance has yet to be developed. Therefore, since molecular delivery by electroporation involves a permeabilization step and a transport step, we hypothesized that splitting the pulses into two separate components for permeabilization and electrokinetically-mediated transport may lead to enhanced delivery efficiency and cell viability.

Our experimental and theoretical analysis of transport during electroporation of
single cell indicates that the cellular influx of species is significantly affected by the residual electric field post-permeabilization, which drives electrophoretic transport and FASS [63, 64]. The residual electric field following electroporation is a critical factor in effective transport in typical electroporation protocols. Electrokinetic theory predicts that exposing a cell to two (or more) pulses - a sharp, high strength pulse to permeabilize the cell, followed by a lower, broader pulse(s) to drive transport and FASS – can significantly improve delivery efficiency, and potentially viability, by removing the need for a substantial residual field and thereby decreasing the overall field exposure. This prediction is supported by various studies in the literature [41, 98, 103, 114, 115]. For instance, Sukharev et al. found that the transfection efficiency can be increased by applying double pulses and by decreasing the gap duration between the double pulses [103]. Satskauskas et al. observed an improved transfection following exposure of the tibialis cranialis muscle of mice to combinations of high and low strength electric pulses in the presence of plasmid DNA with a reporter protein [114]. Guignet and Meyer showed that the best combination of delivery efficiency and viability in vitro occurred for two, smaller pulses rather than one large pulse [41]. Smith and co-authors modeled the double pulse experiments from Sukharev et al. [103] and stated that in addition to the significant role of electrophoretic mediated transport, delivery was also dependent on the pore dynamics during electroporation [98]. Several other authors have demonstrated the importance that pore dynamics play in adjusting the electrophoretic transport [123]. In more recent work, Stroh et al. demonstrated empirically the improved efficiency of delivery for RNA and DNA into various types of cells without providing any physical insight [101].

In this work, the delivery and cellular viability occurring via double pulses with different second pulse field strength and duration, but with a similar first pulse, are compared to each other. The delivery and viability are scaled with respect to the field strengths and durations and a critical regime is defined where both of them are maximized. Flow cytometry was used to measure the fluorescence signal for both delivery and viability. In addition, the fast acquisition of large number of cells provides a statistically significant data. Fluorescein-dextran (FD) (MW ~ 10,000 Da), which has limited entry after pulse
application, was utilized in this study to quantify the amount uptaken into the cell. On the other hand, viability was analyzed using 7-Aminoactinomycin (7-AAD). By demonstrating the importance of electrokinetic transport, while simultaneously characterizing delivery efficiency and viability based on physical parameters for reliable electroporation, these protocols will be applied in vitro in a novel on-chip micro-electroporation device, and towards the design of in vivo protocols in future studies.

1.4 Thesis Structure

This thesis proceeds as follows:

In chapter 2, we systematically characterized the electrodeformation of vesicles with higher conductivity ratios. Unilamellar vesicles were electroformed with prescribed intra-to-extra-vesicular conductivity ratios ranging from 1.92-53 and pulsed by electric fields of different strengths. We quantified and analyzed the aspect ratio, volume, and surface area during and after pulsation. We developed the first comprehensive model to quantitatively predict large vesicle electrodeformation. This study elucidated the electrodeformation mechanics involved in a complex domain where strong, non-linear deformation is coupled with a significant poration of the membrane. This work has been summarized in a publication in Physical Review E journal [93].

In chapter 3, we designed and implemented experiments to study the complex transport mechanisms and to quantify the molecular uptake in electroporation-mediated molecular delivery. Specifically, we investigated the temporal and spatial evolution of propidium iodide (PI) during and after pulse application for six buffers with different electrical conductivity. We quantified the respective contributions of electrophoresis and diffusion and identified a critical electrokinetic mechanism (FASS) that influences the achievable intracellular concentration into the cell. The experimental quantification efforts are compared directly with model predictions. The results offer significant physical insights for the understanding of the transport processes involved during electroporation. This work has been summarized in a publication in Biochimica et Biophysica Acta (BBA) - Biomembranes [94].
In chapter 4, the molecular delivery and cell viability using double pulse electroporation were investigated. Since molecular delivery by electroporation involves a permeabilization step and a transport step, we split the pulses into two separate components and optimized their duration and strength in order to enhance delivery efficiency and cell viability. We built a two-stage electroporation device that delivers separate pulses for electroporation and electrokinetically-mediated transport. The delivery of dextrans and the viability of 3T3 cells were analyzed with flow cytometry. The effects of field strength and pulse duration were analyzed and quantified. A critical regime was defined based on a parametric analysis. This work aimed to develop a general methodology based on physical principals, as opposed to the current protocols that are *ad hoc* and empirical in nature.

The main conclusions of this dissertation are summarized and presented in chapter 5.
Chapter 2

Vesicle Deformation and Poration Under Strong DC Electric fields

2.1 Introduction

In this chapter, we present both an experimental and a theoretical analysis for the electrodeformation of giant lipid vesicles under strong DC electric fields. The electrodeformation is investigated with direct optical observation. Unilamellar vesicles 14-30 \( \mu \text{m} \) in diameter were formed from L-\( \alpha \)-phosphatidylcholine [2], with prescribed intra-to-extra-vesicular conductivity ratios ranging from 1.92-53.0. Direct-current (DC) pulses 500 \( \mu \text{s} \) in duration and 0.9-2.0 kV/cm in strength were applied to the suspended vesicles. This arrangement led to strong prolate deformations in which the vesicles elongated along the direction of the applied field. The vesicles were visualized using sucrose-glucose contrast imaging. The deformation was quantified by measuring the aspect ratio of the deformed vesicles, and the changes in the apparent surface area and volume are analyzed both during and after pulsation. The experimental results are compared with theoretical and modeling work.

Previous work primarily investigated the time course of deformation (in DC fields, [89, 90]), and characterized the various morphological types and the transition from one to another (in both AC and DC fields, [4, 22, 90, 112]). Herein, we focus on one particular morphological type, namely, the prolate deformation observed under DC electric pulses. We systematically quantify deformation as a function of the controlling parameters, i.e., the field strength, and the intra-to-extracellular conductivity ratio. In contrast to the moderate-deformation regimes (with aspect ratios not exceeding 3) studied previously [89, 90], this work extends into a strong-deformation regime. In some conditions, the combination of high field strength and conductivity ratio results
in an aspect ratio exceeding 10. Electrodeformation of this magnitude has not been previously reported. Meanwhile, appreciable changes in vesicle surface area and volume are also present, which is consistent with observations by Portet et al. [81]. These changes indicate the concurrence of electroporation. The phenomena examined in this work therefore represent a complex new domain where strong, nonlinear deformation is coupled with significant poration of the membrane.

The wide range of data provided by this work is valuable for validating and advancing current understanding, in particular through simultaneous model development. Therefore, we pursue a predictive theory to interpret the experimental results. Our model directly extends from that by Hyuga et al. [49, 50]. In contrast to all previous theories which are limited to the linear, small-deformation regime [49, 50, 59, 112, 118], the current model is able to predict large membrane electrodeformations. The model results are compared with the experimental data, which reveals both agreement and discrepancies. In particular, the agreement suggests the dominating role of the electrostatic force in driving the deformation. The discrepancies, on the other hand, point to model limitations. These limitations are discussed together with suggestions for improvements. The theoretical work was accomplished with the help of Dr. Jianbo Li. This work has been published in the Physical Review E journal [93].

2.2 Materials and Methods

2.2.1 Preparation of Vesicles

Unilamellar vesicles were formed using an electroformation technique developed by Angelova and Dimitrov [2]. L-α-phosphatidylcholine (from egg-PC, Sigma, St. Louis, MO) was dissolved at an approximate concentration of 2 mg/mL in a pre-mixed chloroform/methanol (9:1, v:v) solution. Three drops of the lipid solution (5 µL each) were deposited on an indium-tin-oxide (ITO) glass slide, which was subsequently dried in a vacuum chamber to remove traces of the organic solvents. A 2.5-mm thick PDMS spacer with a rectangular open space in the middle was placed on the lipid-coated slide,
and covered by another ITO slide to form the electroformation chamber. The layered structure was integrated into a resin frame for sealing and handling. Sucrose and sodium chloride (NaCl) were dissolved in de-ionized (DI) water at defined concentrations (Table 2.1), and the solution was injected into the sealed chamber. An AC electric field, 2 V/mm (RMS) in amplitude and 10 Hz in frequency, was applied through the conductive ITO slides for 40 minutes (Fig. 2.1). A population of vesicles ranging from 14-30 µm in diameter formed due to this process.

2.2.2 Separation of Vesicles

The vesicles were aspirated gently from the electroformation chamber. The vesicle-liquid mixture was filtered with a syringe filter to remove most of the liquid. The remaining portion was then washed and re-suspended in DI water containing a desired glucose concentration, which generated a difference in the intra-vesicular (sucrose) and extra-vesicular (glucose) solutions. (The solution within the vesicle is assumed to have the same properties as those of the liquid in the electroformation chamber.) This sugar asymmetry allowed imaging of the vesicle due to the contrast in the index of refraction inside and outside of the vesicle [4, 22, 89, 90].
Table 2.1: The intra-to-extra-vesicular conductivity ratio ($\gamma$) was varied by controlling the conductivity inside ($\lambda_{in}$) and outside ($\lambda_{out}$) of the vesicles.

<table>
<thead>
<tr>
<th>$\gamma$</th>
<th>$\lambda_{in}$ ($\mu$S/cm)</th>
<th>$\lambda_{out}$ ($\mu$S/cm)</th>
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<tbody>
<tr>
<td>1.92</td>
<td>6.20</td>
<td>3.23</td>
</tr>
<tr>
<td>5.97</td>
<td>18.7</td>
<td>3.13</td>
</tr>
<tr>
<td>32.6</td>
<td>112</td>
<td>3.44</td>
</tr>
<tr>
<td>46.9</td>
<td>138</td>
<td>2.94</td>
</tr>
<tr>
<td>53.0</td>
<td>138</td>
<td>2.60</td>
</tr>
</tbody>
</table>

2.2.3 Electrical Conductivity/Osmolality Measurements

The electrical conductivities were controlled by altering the NaCl concentration in the intra- and extra-vesicular solutions to probe the effects of this variable on the characteristics of vesicle deformation. The intra-vesicular conductivity ranged from 6.20 $\mu$S/cm (with no added salt) to 138 $\mu$S/cm (with 1 mM NaCl). Other conductivities between these values were achieved by increasing the salt concentration gradually. The conductivity of the glucose solution suspending the vesicles ranged from 2.60 $\mu$S/cm to 3.44 $\mu$S/cm. All conductivity values (Table 2.1) were measured with a conductivity meter (CON 6, Oakton Instruments, Vernon Hills, IL). The osmolalities of the intra- and extra-vesicular solutions were also carefully measured (3D3 Osmometer, Advanced Instruments, Norwood, MA), and matched by moderating the glucose concentration in the extra-vesicular solution to avoid effects due to osmotic pressure.

2.2.4 Vesicle Electrodeformation/Electroporation and Imaging

To perform electrodeformation studies, approximately 70 $\mu$L of vesicle-containing solution was placed in a separate chamber consisting of two stainless steel electrodes ($D = 0.61$ mm) affixed to a microscope slide with a separation distance of 2.75 mm. An electroporator was custom-built to deliver calibrated, controllable, square pulses 300-5000 $\mu$s in duration and 0-700 V in amplitude (Note: By our specific design, the voltage amplitude and pulse shape did not depend on the conductivity of the vesicle-containing solution). The electroporator was synchronized with an imaging system through a timing box (Model 535 Delay Generator, Berkeley Nucleonics, San Rafael,
The imaging system consisted of a digital camera (Hamamatsu C4742-95, Bridgewater, USA) connected to an inverted microscope (Olympus IX81, Center Valley, PA). The integrated system allowed the recording of a single image at a defined delay time after the electric pulse started.

The vesicles were visualized using phase-contrast microscopy. For each experiment, three snapshots of an isolated vesicle were acquired. 1) A reference image was taken before the application of an electric pulse to capture the original (spherical) shape and size of the vesicle (Fig. 2.2(a) and Fig. 2.3(a)). 2) After the application of a DC electric pulse, a second image was taken at a defined delay time (with respect to the start-time of the pulse), using the synchronization scheme described above. This image captured the deformed vesicle toward the end of the electric pulse (Fig. 2.2(b) and Fig. 2.3(b)). 3) A final image of the same vesicle was taken a few seconds post-pulsation. The delay was sufficient for the vesicle to relax back to a spherical shape, but with possible membrane loss and size reduction in some cases (see Fig. 2.2(c) and Fig. 2.3(c)). All images were post-analyzed using NIH ImageJ.

2.3 Experimental Results

In a DC electric field, the morphological type of a deformed vesicle is controlled by its intra-to-extravesicular conductivity ratio \([89, 90]\), which we henceforth denote by \(\gamma\). For example, \(\gamma > 1\) induces a prolate deformation where the vesicle elongates along the direction of the applied field, whereas \(\gamma < 1\) induces an oblate deformation where the vesicle is compressed along the field vector. These behaviors can be explained by the fact that different \(\gamma\) configurations lead to different directions of the electrostatic force on the membrane \([89, 90]\).

2.3.1 Prolate Electrodeformation of Vesicles

In this work, we focus on the prolate regime (\(\gamma > 1\)). Vesicles were prepared with five intra-to-extra-vesicular conductivity ratios as described previously, namely, \(\gamma = 1.92, 5.97, 32.6, 46.9, \) and \(53.0\) (Table 2.1). For each conductivity ratio, three electric field
Figure 2.2: Bright-field images of a vesicle before (a), during (b), and after (c) the application of an electric pulse. The vesicle is originally $D_i = 25.9$ µm in diameter. The applied field is $E = 0.9$ kV/cm and pointing from right to left. The conductivity ratio is $\gamma = 53.0$. The shape of the vesicle during application of the electric field is fitted with an ellipse (black dashed line) using ImageJ (b). The vesicle demonstrates a moderate prolate deformation with an aspect ratio of $p = 1.31$. No obvious size reduction is observed in the post-pulsation image (c).

A series of electrodeformation experiments, 85 in total, were performed in this study. Representative deformations and recoveries are shown in Figs. 2.2 and 2.3. Fig. 2.2(a) shows a pre-pulsation vesicle, with an initial radius (denoted by $D_i$) of 25.9 µm. The conductivity ratio was $\gamma = 53.0$. Fig. 2.2(b) shows the same vesicle at the end of an electric pulse, with the direction oriented from right to left, and a magnitude of 0.9 kV/cm. The geometric shape was well-fitted with an ellipse (black dashed line). An aspect ratio, $p$, defined as the ratio of the major and minor axes of the fitted ellipse, is used to quantify the degree of deformation (Fig. 2.2(b)). For the case shown in Fig. 2.2(b), $p = 1.31$. As seen in Fig. 2.2(c), the vesicle relaxed back to a spherical shape approximately 2 seconds post-pulsation. The diameters before and after pulsation are obtained by similarly fitting the vesicles in each image with circles (not shown). No apparent membrane loss or size reduction was observed in the case shown in Fig. 2.2.

In contrast, Fig. 2.3 shows a stronger deformation during the electric pulse (Fig.
Figure 2.3: Bright-field images of a vesicle before (a), during (b), and after (c) the application of an electric pulse. The vesicle was originally $D_i = 28.8 \, \mu m$ in diameter. The applied field was $E = 1.5 \, kV/cm$ and pointing from right to left. The conductivity ratio was $\gamma = 46.9$. The shape of the vesicle during application of the electric field is fitted with an ellipse (black dashed line) using ImageJ (b). The vesicle demonstrated a large prolate deformation with an aspect ratio of $p = 3.57$. The polar caps are not clearly visible in (b), possibly due to membrane poration. The post-pulsation image (c) shows an appreciable reduction in vesicle size.

2.3(b)), and an appreciable shrinkage after the pulse is switched off (Fig. 2.3(c)). The initial diameter was $D_i = 28.8 \, \mu m$, the conductivity ratio was $\gamma = 46.9$, and the electric field strength was $E = 1.5 \, kV/cm$ (also pointing from right to left). Although the polar caps are not clearly visible for the deformed vesicle in Fig. 2.3(b), possibly due to a loss of membrane, or a temporary loss of the sucrose/glucose contrast in the proximity of the poles induced by membrane permeabilization, the shape is still well-fitted with an ellipse ($p = 3.57$). We emphasize that in this and other similar cases, the vesicles may have local irregularities (especially toward the polar areas) causing deviations from ellipsoidal shapes. The fitted ellipse is therefore an estimate of the realistic vesicle geometry. However, we believe that such a fitting is a reasonable approximation. Similar situations occur, e.g., in [89] (see Fig. 5 therein), where it can be more clearly seen that although the polar boundaries become fuzzy and less visible, the proper vesicle is still in general ellipsoidal. Alternatively, we have attempted to obtain contour fitting of the vesicles without any presumption on their shape (data not shown). When compared with results from elliptical fitting, this more direct and precise approach reveals differences of around 1% for the aspect ratio, and 2-4% for surface area and volume (examined in Fig. 2.6 below). We therefore opt to adopt the elliptical fitting method based on its simplicity and in consideration of the large amount of data involved. In Figure 2.3(c), the post-pulsation image shows obvious size reduction when compared with Fig. 2.3(a).
Figure 2.4: The aspect ratio of deformed vesicles ($p$) induced by three electric field strengths: (a) 0.9 kV/cm, (b) 1.5 kV/cm, and (c) 2.0 kV/cm. The pulse width was 500 µs for all the experiments. Each data point represents a single experiment. The values for $p$ are obtained from analyzing images in the same manner as in Figs. 2.2(b) and 2.3(b).

The membrane loss was possibly caused by one of the three mechanisms discussed by Portet et al. [81].

### 2.3.2 Aspect Ratio Analysis

In Figure 2.4, the aspect ratio, $p$, of the deformed vesicles is plotted against the initial vesicle diameter, $D_i$, for the three field strengths studied. Different geometrical symbols represent different $\gamma$ values. At the relatively low field strength ($E = 0.9$ kV/cm), the deformation aspect ratio is around 2 for all conductivity ratios, as seen in Fig. 2.4(a). As the field strength is increased to 1.5 kV/cm (Fig. 2.4(b)), higher deformation aspect ratios (exceeding 4) are observed, and are accompanied by a more obvious dependence on $\gamma$. At the highest field strength ($E = 2.0$ kV/cm), very large deformations with values of $p$ approaching 13 are present, in particular at the highest conductivity ratio, $\gamma = 53.0$. For all cases studied, no obvious dependence of $p$ on $D_i$ is observed.
Figure 2.5: (a) The average aspect ratio, $<p>$, as a function of the conductivity ratio, $\gamma$. The error bar represents the standard deviation of each subset. The parameters for the exponential fits (Eqn. (2.1), dashed) are listed in Table 2.2. (b) The fitted constant $p_0$ exhibits a linear scaling with $E^2$. The dashed line is a least-square linear fit, $p_0 = 1.76E^2$. The coefficient of determination is $R^2 = 0.93$.

In Fig. 2.5(a), the systematic dependence of the deformation ratio on the conductivity ratio, $\gamma$, and the field strength, $E$, is examined. Here we define an average deformation ratio, $<p>$, which is calculated by taking the average of all values of $p$ in each subset of vesicles with identical conductivity ratio and field strength. The error bars indicate standard deviation. The deformation ratio has a positive correlation with the electric field, which has been indicated by Fig. 2.4. The dependence of $<p>$ on $\gamma$ shows a rapid rise followed by a relative plateau. The data is fitted with an exponential function of the form:

$$<p> = p_0(1 - e^{\frac{1-\gamma}{\gamma_p}}) + 1$$  \hspace{1cm} (2.1)$$

which is shown as dashed lines in Fig. 2.5(a). The fitting parameters $p_0$ and $\gamma_p$ for each data set are given in Table 2.2. Importantly, the constant exhibits a quadratic scaling with $E$ (Fig. 2.5(b)), indicating the dominant role of the Maxwell stress in driving the deformations.

2.3.3 Volume and Surface Area Analysis

Associated with the morphological changes, the vesicles also experienced dynamic changes in the apparent surface area and volume. The changes are analyzed by the following
Table 2.2: Constants from the exponentially fitted data using Eqn. (2.1). The fitted curves are shown in Fig. 2.5(a).

<table>
<thead>
<tr>
<th>$E$ (kV/cm)</th>
<th>$p_0$</th>
<th>$\gamma_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>0.78</td>
<td>1.20</td>
</tr>
<tr>
<td>1.5</td>
<td>2.96</td>
<td>2.02</td>
</tr>
<tr>
<td>2.0</td>
<td>7.73</td>
<td>6.72</td>
</tr>
</tbody>
</table>

formulae:

\[
\alpha_d = \frac{(A_d - A_i) \times 100}{A_i}, \quad \varpi_d = \frac{(V_d - V_i) \times 100}{V_i} \quad (2.2)
\]

\[
\alpha_f = \frac{(A_f - A_i) \times 100}{A_i}, \quad \varpi_f = \frac{(V_f - V_i) \times 100}{V_i} \quad (2.3)
\]

Here $\alpha$ and $\varpi$ denote the relative percentage change in the apparent surface area ($A$) and volume ($V$) of each vesicle, and the subscripts $i$, $d$, $f$ denote “initial”, “during”, and “final”, respectively. All of $A_i$, $A_f$ and $V_i$, $V_f$ are calculated assuming the vesicle is spherical, and the radius is estimated from a fitting on images similar to Fig. 2.2(a), (c) and Fig. 2.3(a), (c) (showing the initial and final stages of the vesicle). $A_d$ and $V_d$ are calculated to capture the changes during field-application, also from a fitting of the images. As we previously mentioned, the vesicle is assumed to be an ellipsoid and is axisymmetric with respect to the direction of the applied field.

Figure 2.6(a) and (b) demonstrate that vesicle deformation during an applied electric field is associated with surface area dilation ($\alpha < 0$) and volume reduction ($\varpi < 0$). In the figure, cyan, red, and black denote the field strengths of 0.9, 1.5, and 2.0 kV/cm, respectively. Different symbols represent different conductivity ratios. Both the surface area and volume vary approximately linearly with respect to the deformation ratio, $p$. Based on the data, the surface area increases with increasing field strength and conductivity ratio. However, this increase in surface area is accompanied by a volume reduction. These trends provide strong indication of membrane poration. When a spherical vesicle becomes ellipsoidal, and assuming that the volume does not initially change, the surface area has to increase, possibly via both the unfolding of the excess
Figure 2.6: Percentage change in the apparent membrane surface area ($\alpha$) and vesicle volume ($\varpi$) during (subscript $d$) and after (subscript $f$) pulsation, and as a function of the aspect ratio, $p$. In the legend, the numbers (e.g., $1.92/0.9$) denote combinations of $\gamma/E$, with $E$ in units of kV/cm. The surface area increases during pulsation and then decreases after the pulse is ceased. The volume decreases consistently throughout the process.

area and elastic stretching of the membrane [70, 89]. Further deformation beyond the compensation of these mechanisms can only occur via leaking of the (incompressible) fluid contained, namely, by means of electroporation. Volume reduction is therefore a natural consequence of large deformations observed in the current experiments. An increase in the apparent area, $A_d$, on the other hand, is speculated to be a combination of the unfolding effects of the excess area, the presence of pores, and the thinning and structure rearrangements of the membrane [70, 102].

Figure 2.6(c) and (d) show the concurrence of area and volumetric reduction ($\alpha_d$, $\varpi < 0$) after the vesicles recovered to the final, spherical shape post-pulsation. The correlations of $\alpha_d$ and $\varpi_d$ with $p$ are similarly monotonic, with some vesicles losing up to 60% of surface area and almost 70% of volume. For the case presented in Fig. 2.3, the membrane surface area loss was 27%, and the volume reduction was 37% by our calculation. When compared with Fig. 2.6(b), Fig. 2.6(d) demonstrates further volume reduction during the recovery process. This observation confirms that pore-sealing was not immediate after the field was switched-off, and further fluid leakage
occurred during this phase. The results corroborate well with those by Portet et al. [81], which proposes that membrane loss is induced by the formation of (small) vesicles, tubules, and/or pores. (However, note that in [81], a similar degree of loss is achieved with multiple pulses, not a single pulse as in the present study.)

2.4 Theoretical Results

The phenomenon studied in this work represents a regime where the complex physics of electrodeformation and electroporation are highly coupled. A comprehensive model including all detailed physical processes, namely, permeabilization, fluid motion and leakage, and large deformation is currently not available. Specifically, all existing theories are limited to the linear regime where the deformation is assumed to be small, and cannot be applied to the case studied in this work [50, 49, 112, 118]. Here we pursue a predictive model for large deformations by directly extending from previous work by Hyuga et al. [50], where the authors studied the effects of a permeabilized, conducting membrane, in contrast to others who consider deformation in the non-permeabilized regime. Hence their framework is more appropriate for the current case. In the theory described below, we adopt the same physical principles as outlined by Hyuga et al. [50] to derive the equation of motion for the deforming membrane. However, instead of solving the problem in the linearized, small-deformation regime using spherical harmonics, we adopt a rotational spheroidal coordinate system to allow for the prediction of arbitrarily large aspect ratios.

Our primary assumption is that the vesicle remains ellipsoidal in shape, thereby ignoring all other shape modes. This approximation follows from our experimental data analysis, and is supported by many experimental observations [4, 22, 89, 90, 112, 118]. A similar treatment for large electrodeformations of droplets is found, for example, in Bentenitis and Krause [6]. For this case, the rotational spheroidal coordinate system is suitable to describe the geometry. The coordinates $(\xi, \eta)$ are related to a cylindrical
system \((\rho, z)\) as:

\[
\begin{cases}
  z = c\xi_n \\
  \rho = \sqrt{x^2 + y^2} = c\sqrt{(\xi^2 - 1)(1 - \eta^2)}.
\end{cases}
\] (2.4)

Here \(z\) and \(\rho\) denote the axial and radial coordinates, respectively, \(c\) is a constant, and is taken to be \(c = \sqrt{a^2 - b^2}\) for an ellipsoid with axes \(a\) and \(b\) \((a > b)\). In this system, the surface of the ellipsoid is conveniently given as:

\[
\xi = \xi_0 \equiv \frac{a}{c} = \frac{a}{\sqrt{a^2 - b^2}}
\] (2.5)

For the derivation below, we also define a shape factor, \(\Theta\), which is related to \(\xi_0\) and the aspect ratio \((p = \frac{a}{b})\) as:

\[
p = \frac{1}{\cos(\Theta)}, \quad \xi_0 = \frac{1}{\sin(\Theta)}
\] (2.6)

Following Hyuga et al. [50], we further assume that the total vesicle surface area, \(S\), is conserved:

\[
S = \int_{-1}^{1} 2\pi ab[1 - \left(\frac{\xi^2}{a^2}\right)\eta^2]^\frac{1}{2}d\eta = 4\pi a_0^2,
\] (2.7)

where \(a_0\) is the radius of the un-deformed, spherical vesicle. We subsequently obtain:

\[
a = a_0\sqrt{\frac{2\tan(\Theta)}{\sin(\Theta)\cos(\Theta) + \Theta}}, \quad b = a_0\sqrt{\frac{2\sin(\Theta)\cos(\Theta)}{\sin(\Theta)\cos(\Theta) + \Theta}}
\] (2.8)

Under the constraint 2.7 and the ellipsoidal assumption, the vesicle geometry is completely characterized by a single parameter, \(\Theta\). We emphasize that the surface area conservation is an idealizing assumption to make the model tractable. Further improvement of the model needs to consider area expansion as indicated by Fig. 2.6.

For the electrical problem, we assume that the Ohmic current is conserved, and that the membrane is permeabilized and conductive:

\[
\nabla \cdot \lambda_{in} \nabla \phi_{in} = \nabla \cdot \lambda_{out} \nabla \phi_{out} = 0
\] (2.9)
\[ \mathbf{n} \cdot \lambda_{in} \nabla \phi_{in} = \mathbf{n} \cdot \lambda_{out} \nabla \phi_{out}, \quad \mathbf{t} \cdot \nabla \phi_{in} = \mathbf{t} \cdot \nabla \phi_{out}, \] at the membrane \hfill (2.10)

where \( \phi \) is the electrical potential, and \( \mathbf{t} \) and \( \mathbf{n} \) denote the unit tangential and normal vector on the membrane, respectively. Eqns. (2.9, 2.10) can be solved in a spheroidal coordinate system. The result pertinent to the current work is the normal component of the Maxwell stress, \( F_n \):

\[
F_n = \mathbf{n} \cdot (\mathbf{T}^{\text{out}} - \mathbf{T}^{\text{in}}) \cdot \mathbf{n}, \quad \mathbf{T} = \varepsilon (\mathbf{E} \mathbf{E} - \frac{1}{2} |\mathbf{E}|^2 \mathbf{I}) \hfill (2.11)
\]

where \( \mathbf{T} \) is the Maxwell stress tensor, \( \mathbf{E} \) is the local electric field vector, \( \mathbf{I} \) is a unit tensor, and \( \varepsilon \) is the permittivity of water. A straightforward calculation reveals:

\[
F_n = \frac{\varepsilon}{2} E^2 B^2 \left( \xi_0^2 - 1 \right) \left( \gamma^2 - 1 \right) \left( \frac{\eta^2}{\xi_0^2 - \eta^2} \right), \hfill (2.12)
\]

\[
B = \frac{Q_1(\xi_0) - \xi_0 Q_1'(\xi_0)}{\gamma Q_1(\xi_0) - \xi_0 Q_1'(\xi_0)}, \hfill (2.13)
\]

where \( E \) is the strength of the applied field, \( Q_1 \) is a Legendre function of the second kind and order one, and \( Q_1' \) denotes its derivative.

The equation of motion follows a Lagrange formulation:

\[
\frac{d}{d\tau} \left( \frac{\partial L}{\partial \dot{\Theta}} \right) - \frac{\partial L}{\partial \Theta} = \int \left( F_n + F_\mu \right) \left( -\frac{\varepsilon}{\sin^3(\Theta)} \sqrt{1 - \eta^2 \sin^2(\Theta)} \right.
\]

\[
+ a_0 \frac{\Theta (2 \cos^2(\Theta) + 1) + \cos(\Theta) \sin(\Theta)}{\sqrt{2 \cos(\Theta) \sin(\Theta) (\cos(\Theta) \sin(\Theta) + \Theta)^2 (1 - \eta^2 \sin^2(\Theta))}} \right) dS. \hfill (2.14)
\]

Here \( L = K - V \) is a Lagrangian function, where \( V \) is the curvature-elastic energy, \( K \) is the effective kinetic energy, \( F_n \) is the normal component of the electrostatic force defined above, \( F_\mu \) is an effective viscous force, and the factors in the parenthesis on the right-hand-side represent a virtual displacement with respect to the single independent
variable, $\Theta$. The evaluation of each individual term is introduced below.

The curvature-elastic energy, $V$, is given by the formula:

$$V = \frac{\kappa}{2} \int (H - H_0)^2 dS,$$

where $\kappa$ is the curvature-elastic modulus, $H$ is the mean curvature, and $H_0$ is its equilibrium value. For a lipid bilayer membrane, we set $H_0 = 0$. Under the current coordinate system, the curvature is given as:

$$H = \frac{\cos^2(\Theta) + 1 - \eta^2 \sin^2(\Theta)}{(1 - \eta^2 \sin^2(\Theta))^{\frac{3}{2}}b},$$

and the integrated energy is evaluated to be:

$$V = \frac{\kappa a \pi}{3b \sin(\Theta)} (14 \cos(\Theta) \sin(\Theta) + 6\Theta + 4 \cos^3(\Theta) \sin(\Theta))$$

The effective kinetic energy, $K$, and the effective viscous force, $F_\mu$, are calculated with an empirical model, also following Hyuga et al. [50]:

$$K = \frac{1}{2} \int \rho_m \nu_n^2 dS, \quad F_\mu = -\mu \nu_n.$$  \hspace{1cm} (2.18)

Here $\rho_m$ is an effective mass density (per unit area of the membrane), $\mu$ is an effective viscous coefficient, and $\nu_n$ is the normal component of the velocity for a point on the deforming membrane:

$$\nu_n = \frac{b \sin(\Theta) \eta^2 + b' \cos(\Theta)}{\sqrt{1 - \eta^2 \sin^2(\Theta) \cos(\Theta)}} \dot{\Theta}.$$  \hspace{1cm} (2.19)

Here a prime denotes a derivative with respect to $\Theta$, and an over-dot denotes a derivative with respect to time. The details of this empirical model are found in [50].

Finally, substituting Eqns. (2.12), (2.17), (2.18) into Eqn. (2.14), we obtain the equation of motion in terms of $\Theta$:

$$2K_0 \ddot{\Theta} + K'_0 \dot{\Theta}^2 + V' - Q_\mu \dot{\Theta} + Q_F = 0.$$  \hspace{1cm} (2.20)
Table 2.3: List of model parameters.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Value</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rho_m$</td>
<td>$8.63 \times 10^{-3} \text{ g cm}^{-2}$</td>
<td>Effective mass density [50]</td>
</tr>
<tr>
<td>$a_0$</td>
<td>$11.3 \mu m$</td>
<td>Initial vesicle radius</td>
</tr>
<tr>
<td>$\mu$</td>
<td>$44.3 \text{ g cm}^{-2} \text{ s}^{-1}$</td>
<td>Effective viscous coefficient</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>$7.17 \times 10^{-10} \text{ F m}^{-1}$</td>
<td>Permittivity of water</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>$5 \times 10^{-20} \text{ J}$</td>
<td>Curvature-elastic modulus [50, 112]</td>
</tr>
<tr>
<td>$t_p$</td>
<td>$500 \mu s$</td>
<td>Pulse length</td>
</tr>
</tbody>
</table>

Figure 2.7: A comparison between theoretical prediction (dashed) and experimental data (symbols). The data are the same as those in Fig. 2.5(a). The model results are generated with $a_0 = 11.30 \mu m$, (a) $\mu = 44.3 \text{ g cm}^{-2} \text{ s}^{-1}$, and (b) $\mu = 22.5 \text{ g cm}^{-2} \text{ s}^{-1}$.

Here $K_0$, $Q_\mu$, and $Q_F$ are all functions of $\Theta$, and their expressions are given in Appendix A. In Eqn. (2.20), the first two terms represent the effects due to inertia, the third arises from the membrane elastic response, and the last two are due to the viscous and the electrostatic forces, respectively. Equation (2.20) is solved numerically using parameters listed in Table 2.3, and for the various $\gamma$ and $E$ values studied in the experiments. For each case, the value for $\Theta$ at the end of a 500 $\mu s$ pulse (the duration used in the experiments) is obtained and the value for $p$ is subsequently calculated using Eqn. (2.6).

The general dependence of $p$ on $\gamma$ and $E$ is well-predicted by the model. The results are compared with the experimental data in Fig. 2.7(a), which reveals good agreement. In the model, we set $a_0 = 11.30 \mu m$, which is the average radius of the vesicle population we studied in the experiments. The trends can be explained by an analysis of the electrostatic force, $F_n$. From Eqn. (2.1), the quadratic dependence of $F_n$ on the applied field strength, $E$, is evident (see also Fig. 2.5(b)). In addition, the
force scales with the conductivity ratio as:

$$F_n \sim \frac{\gamma^2 - 1}{[\gamma Q_1(\xi_0) - \xi_0 Q_1'(\xi_0)]^2} \quad (2.21)$$

For small values of $\gamma$, and temporarily treating both $Q_1(\xi_0)$ and $\xi_0 Q_1'(\xi_0)$ as constants, Eqn. (2.21) is simplified to:

$$F_n \sim \frac{\gamma^2 - 1}{[\xi_0 Q_1'(\xi_0)]^2} \quad (2.22)$$

This quadratic relationship explains the initial rise in $p$ with respect to $\gamma$. For large values of $\gamma$, Eqn. (2.21) converges to a constant, which explains the plateaus observed in Fig. 2.5(a).

Despite the agreement shown in Fig. 2.7(a), the model can appreciate further improvement, through the development of a more rigorous framework to predict the physical processes involved. A main limitation of the current theory is that the hydrodynamic problem is treated empirically, with a free parameter, the effective viscous coefficient $\mu$, (Eqn. (2.18)). In fact, if we decrease the value for $\mu$ to the largest value used by Hyuga et al. [50] (22.5 g cm\(^{-2}\) s\(^{-1}\)), the model overpredicts the deformation ratios (Fig. 2.7(b)). In addition, there is a discrepancy concerning the dependence of the aspect ratio on vesicle size. Fig. 2.8 shows the deformation ratio as a function of the initial diameter, $D_i$. In contrast to the data in Fig. 2.4, where no obvious dependence on $D_i$ is observed, the model predicts that $p$ decreases with an increasing $D_i$. This is because in Eqn. (2.20) the coefficients $K_0$ (representing the effects of inertia) and $Q_\mu$ (representing the viscous effects) scale as $a_0^4$, where $a_0 = \frac{D_i}{2}$, whereas the coefficient $Q_F$ (representing the driving electrostatic force) scales as $a_0^3$. Therefore, as $a_0$ decreases, the deformation force becomes more dominant relative to the resisting forces. We speculate that this effect is likewise caused by the lack of rigor in calculating the hydrodynamic forces.

We believe that the fidelity of the model may be improved by solving the Stokes equation for fluid motion, also under the rotational elliptical coordinate system, such as to compute the fluid stress on the membrane in a rigorous manner. This task is the scope of our on-going work. Another model limitation is the idealizing assumption of surface area conservation, which is obviously violated in our experiments (Fig. 2.6). The area
Figure 2.8: Predicted aspect ratio, $p$, as a function of the initial diameter, $D_i$. The $\gamma$ values correspond to those used in experiments (Table 2.1). In each graph, the curves for $\gamma = 32.6, 46.9, 53.0$ follow each other closely. Contrary to the experimental data in Fig. 2.4, the aspect ratio has a noticeable dependence on the initial vesicle diameter, $D_i$.

expansion observed often induces additional elastic responses of the membrane, which need to be properly included in an improved theory.

2.5 Conclusion

In this work, we presented a study of vesicle deformation under strong DC electric fields. We systematically quantified the degree of deformation as a function of the field strength and the conductivity ratio. In particular, we studied large deformations in the prolate regime ($\gamma > 1$) with aspect ratios approaching 13 in extreme situations. We observed that the degree of deformation depends monotonically on the magnitude of the applied electric field. In addition, the degree of deformation also strongly depends on the conductivity ratio, exhibiting a positive correlation followed by a plateau. An estimate of the apparent vesicle surface area suggests significant expansion during pulsation, and reduction after pulsation, during which the vesicles relaxed back to a spherical shape. Concurrently, the vesicle volume decreased throughout the process. These
dynamic changes indicate membrane losses induced by electroporation. Indeed, the transmembrane potential exceeded the critical threshold for electroporation for most of the experiments performed.

We have developed a mechanistic model to predict the deformation process, and to interpret the experimental results. In contrast to all previous theories, which are limited to the linear, small-deformation regime, the current model is able to predict large membrane electrodeformations. A comparison between the model results and the experimental data reveals both agreement and discrepancy. Specifically, the dependence of the aspect ratio on the conductivity ratio and the applied field strength is well-captured by the model, and the data trends can be explained by the behavior of the driving electrostatic force. On the other hand, the model predicts that the aspect ratio decreases along with an increasing vesicle diameter, whereas such trend is not observed in the experiments. This discrepancy points to the limitations of the current model, which can be possibly improved via the development of a more rigorous electrohydrodynamic theory.
Chapter 3
The Effect of Conductivity on Electroporation-Mediated Molecular Delivery

3.1 Introduction

In this chapter, we aim to quantify the transport of small molecules using time- and space-resolved fluorescence microscopy. We investigate the delivery of PI into 3T3 mouse fibroblasts. The spatial evolution of the fluorescence profile is continuously monitored both during and after pulse application, and for six extracellular buffer electrical conductivities. Our work is motivated by the following considerations. First, we intend to quantify the respective contributions of electrophoresis and diffusion. In contrast to the phototube measurement by Pucihar et al., our experiments provide the necessary resolution to track the dynamic pattern of transport mechanism [86]. In addition, we explore a wider parametric range by varying the extracellular conductivity. Second, we aim to tackle the physical processes leading to the systematic behavior from earlier studies, namely the inverse correlation between delivery and extracellular conductivity observed by Zimmerman and co-authors [23, 68]. However, in their work, the fluorescence signal for PI uptake was acquired by flow cytometry minutes after pulsation, therefore did not reveal the spatial and the temporal dynamics of molecular entry. In the supra electroporation (nano-second pulse) study by Müller et al., only the result of the percentage of PI uptake was presented [68]. In addition, in [23], exponentially decaying pulses were used. Because the decay time is affected by and inversely correlated with the buffer conductivity, its effects on delivery could not be separated from those of other contributors [51]. Better controlled experiments are needed to help identify the root cause of the system behavior. Third, we aim to provide data to directly compare with model predictions. In recent model studies conducted by
our research group [63, 64], we discovered that field-amplified sample stacking (FASS), an electrokinetic mechanism arising from the presence of a non-uniform electric field, plays an important role in mediating molecular delivery via electrophoresis [63, 64]. We proposed that this mechanism in part lead to the correlation observed in [23, 68]. Furthermore, we have developed a simple formula to predict the total electrophoretic delivery, which is also a function of the extracellular conductivity. The current work will directly validate this model.

Our experiments are well controlled with pulses that are rectangular in shape, and not affected by the medium conductivity. For each experimental condition 30-50 single cell experiments were performed, and the results were averaged to achieve statistical significance. The analysis of the data reveals that during the pulse, both the peak and the total integrated fluorescence intensity exhibit an inverse correlation with extracellular conductivity. This behavior corroborates with the FASS theory proposed earlier [63, 64]. In this case the non-uniformity of electric field is induced by the conductivity difference between the cytoplasm and the buffer [63]. A detailed account on the theory is found in [63]. Furthermore, the respective contributions from electrophoresis and diffusion have been quantified; the former is shown to be consistently higher than the latter for the experimental conditions considered. The results are compared with a compact model developed in [63, 64] and good agreements are found between the two. The success of the model validates itself as a useful formula to estimate molecular delivery via electrophoresis. In addition, we discovered that cell swelling post-pulsation increases monotonically with the buffer conductivity, despite that our solutions were isotonic. The experimental quantification and modeling tools developed in this work are important contributions toward designing optimized protocols for a wide range of applications utilizing electroporation.
Table 3.1: List of experimental conditions. The extracellular buffer conductivity is denoted by $\sigma_e$.

<table>
<thead>
<tr>
<th>Solution #</th>
<th>MgCl$_2$ (mM)</th>
<th>$\sigma_e$ ($\mu$S/cm)</th>
<th>Total Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>1.1</td>
<td>250</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>2.4</td>
<td>500</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>3.8</td>
<td>750</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>5.2</td>
<td>1000</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>11.2</td>
<td>2000</td>
<td>43</td>
</tr>
</tbody>
</table>

3.2 Materials and Methods

3.2.1 Cell Culture

NIH 3T3 mouse fibroblasts were plated in six-well plates and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with Fetal Bovine Serum (FBS) (10% v/v), 1% Penicillin-Streptomycin (1% v/v), and L-Glutamine (1% v/v) (Sigma, St. Louis, MO) at 37°C and 5% CO$_2$. Cells were harvested at 70-80% confluency using 0.5% trypsin (Sigma) and washed once with DMEM prior to each experiment. For each experiment, 10-15 different cells were electroporated for each condition. All conditions were conducted on the same day from the same passage of cells, and the entire experimental design was repeated three times.

3.2.2 Reagents

The iso-osmotic pulsing buffers were prepared from 250 mM sucrose (Sigma), 10 mM HEPES (Sigma), and various concentrations of MgCl$_2$ salt (Sigma) (Table 3.1). The pH of the buffers was measured with a Beckman $\Phi$ 340 pH/Temp Meter (Beckman Coulter, Inc., Fullerton, CA) and was adjusted to 7.4. The osmolalities and conductivities of the solutions were measured with an Advanced Osmometer Model 3D3 (Advanced Instrument, Norwood, MA) and the CON 6/TDS 6 hand-held Conductivity/TDS Meter (Oakton, Vernon Hills, IL), respectively. The osmolality of each solution was adjusted to 310 mOsm/kg by adding appropriate amounts of sucrose [55]. The values for the conductivity are presented in Table 3.1.
3.2.3 Single Cell Electroporation

After trypsinization for 5 min, cells were washed once with cell culture media to neutralize the trypsin and spun at 2000 rpm (441×g) for 2 minutes (Allegra X-22 series, Beckman Coulter, Miami, FL). The supernatant was aspirated and the cell pellet was washed twice with the desired pulsing buffer, and spun both times at 500 rpm (26×g) for 1 min. The final cell suspension was resuspended in 500 µL of pulsing buffer containing 100 µM of PI (MW ~ 668 Da) (Sigma). To perform electroporation experiments, approximately 30 µL of cells in suspension (10^4 cells/mL) were placed in a custom made electroporation chamber comprising two parallel stainless steel electrodes (D = 0.61 mm) affixed 1 mm apart on a microscope slide. Calibrated, controlled square electric field pulses of 0.8 kV/cm strength and 100 ms pulse-width were applied utilizing a custom-built electroporator. We remark that this pulsing scheme would likely have resulted in significant cell death. However, the immediate purpose of this study is to understand the transport mechanisms during electroporation but not to optimize the process.

3.2.4 Fluorescence Imaging

The electroporator was synchronized through a timing box (Model 535 Delay Generator, Berkeley Nucleonics, San Rafael, CA) with a CCD camera (ProgRes® JENOPTIK MF Cool, JENOPTEK Optical Systems, Inc., Jupiter, FL) attached to an inverted microscope (Olympus IX71, Center Valley, PA). The integrated system allowed image acquisition to begin before the onset of the electric pulse. Images were captured digitally at 40 frames-per-second (fps) for 7 s. The applied electric field was sufficient to permeabilize the cellular membrane and allowed entry of PI into the cell cytoplasm. Upon binding to DNA/RNA, PI emitted fluorescent signal (ex: 536 nm, em: 617 nm), which was quantified to measure the dynamic accumulation of the dye within the cell. For reference, the maximum achievable fluorescence intensity was obtained from saponin treated cells. Cells were treated with 0.05% saponin (Sigma) and incubated on ice for 10 min. The cell suspension was washed twice with Phosphate Buffer Saline
(PBS) without calcium and magnesium ions (Sigma) and incubated on ice for 20 min in PBS solution containing 100 µM of PI. The experiment was performed for a total of 20 cells. For each cell, a bright field image was taken to measure the cell size, and a single fluorescence image was acquired to measure the intensity.

### 3.2.5 Experimental Analysis Methods

All images and data were analyzed with MATLAB (The Mathworks, Inc., Natick, MA). To account for background fluorescence, the intensity from four 20 pixel x 20 pixel regions in the corners of each image, where continuous changes were never observed, was averaged and subtracted from the actual image. The fluorescence intensity profile along the cell centerline for each image was determined and averaged for all cells from the same condition. The total fluorescence intensity (TFI) inside a cell was calculated by integrating the signal over the whole projected cell area and normalized by either the initial cell volume or the final cell volume, since some changes in cell diameter were observed. The TFI was averaged over the total number of cells for each experimental condition listed in Table 3.1. The TFI for saponin treated cells was obtained similarly. The initial and final radii of each cell were determined from bright field images taken before the pulse and 30 s after the pulse ended, respectively.

### 3.3 Results

#### 3.3.1 Spatial and Temporal Analysis of Propidium Uptake

Representative images and centerline profiles of PI spreading in two buffers with different conductivities are shown in Fig. 3.1. The applied electric field was 0.8 kV/cm in strength, 100 ms in length, and pointed from left to right. Fig. 3.1(a) and (b) show the progression of the fluorescence signal inside the cell before the pulse ($t = -5$ ms), during the pulse ($20$ ms $< t < 95$ ms), immediately after the pulse ($t = 120$ ms), and well after the pulse ($t = 5000$ ms). The cell periphery is indicated by white circles. PI initially entered the cell asymmetrically from the anode-facing side along the electric field during the pulse. After the pulse ceased, both spreading within the cell and PI
Figure 3.1: Temporal dynamics of PI entry into electroporated 3T3 mouse fibroblasts. The applied electric field was 0.8 kV/cm in strength, 100 ms in length, and pointed from left to right. Typical progression of the fluorescence signal for two conductivities, (a) $\sigma_e = 100 \, \mu$S/cm and (b) $\sigma_e = 2000 \, \mu$S/cm, respectively. The fluorescence profile along the cell centerline for the cases shown in (a) and (b) are provided in (c) and (d), respectively. The fluorescence intensity for the low conductivity case is higher than that for the high conductivity case at all times.

entry from the cathode-facing side were observed. Fig. 3.1(c) and (d) demonstrate the fluorescence intensity signal across the cell centerline. From the fluorescence images and the corresponding cell centerline plots, it is evident that the peak fluorescence intensity is higher for the low conductivity case ($\sigma_e = 100 \, \mu$S/cm) than for the high conductivity case ($\sigma_e = 2000 \, \mu$S/cm). The rate of increase of the peak fluorescence is greater during the pulse than after the pulse for both conductivities. The differences in the spatial and temporal evolution of fluorescence intensity during and after pulse application indicate that different mechanisms are involved in transporting PI during and post pulsation.

The dependence of the fluorescence intensity on the extracellular conductivity is more clearly demonstrated in Fig. 3.2. In Fig. 3.2 (a), the cell centerline profile at $t = 95$ ms (the last frame before the end of the pulse) is shown for all six buffer conductivities.
Figure 3.2: (a) Average centerline fluorescence intensity profiles at \( t = 95 \) ms (the last frame acquired before the electric field was switched off) for the six buffer conductivities. (b) The peak intensity value is plotted as a function of buffer conductivity, which shows an inverse correlation between the two. Error bars represent standard error.

For each conductivity, the signal was obtained by averaging the centerline profile for all cells in that group. The fluorescence intensity consistently decreased with increasing buffer conductivity. The maximum value is plotted as a function of \( \sigma_e \) in Fig. 3.2(b). The inverse correlation corroborates with findings from previous reports [23, 68].

3.3.2 Cell Size Changes Before and After Electroporation

Figure 3.3 examines electroporation-induced cell swelling. In Fig. 3.3(a), the averaged cell radius both before and well after the pulse application are shown for the different buffer conditions. In Fig. 3.3(b), the difference between the two is plotted. A monotonic increase in the degree of cell swelling as a function of buffer conductivity is observed. The measurement of the cell size is useful in normalizing the total fluorescence intensity, which is studied next.

3.3.3 Total Propidium Uptake During and After Electroporation

Figure 3.4 shows the evolution of the total fluorescence intensity (TFI) as a function of time and extracellular conductivity. The TFI is normalized by either the initial cell volume (Fig. 3.4(a) and (b)) or the final cell volume (Fig. 3.4(c) and (d)). The temporal evolution of the TFI for all six conductivities exhibits three distinguishable stages. The
first stage occurs when the pulse is present (Fig. 3.4(b) and (d)). During this stage, the fluorescence signal increases sharply in a nearly linear fashion. The second stage starts after the pulse ceases and extends until approximately $t = 1500$ ms (Fig. 3.4(a) and (c)). In this phase, the fluorescence continues to increase, but at a slower rate compared to that during the pulse, until it plateaus at around $t = 1500$ ms. The last stage occurs from around $t = 1500$ ms until the end of acquisition at $t = 7000$ ms. During this period, the TFI remains nearly constant (Fig. 3.4(a) and (c)). In general, normalizing by the initial or final radii affected the magnitude of the TFI but not the trend of the results.

The contributions from the respective stages are illustrated in Fig. 3.5. The TFI for all cases were normalized by the average value derived from the 20 saponin treated cells (14,993 (a.u)), which provided a reference for the maximum achievable TFI when all binding sites are occupied. The curves with (■) describe the TFI values at the end of acquisition, which are accumulated from all three stages and represent total delivery. The curves with (▲) designate the TFI at the end of stage one, which represents the contribution from electrophoretic transport. The difference between the two is indicated by (●), which represents the contributions from post-pulsation transport, where diffusion
Figure 3.4: The total fluorescence intensity (TFI) as a function of time and extracellular buffer conductivity. (a) and (b) the TFI is normalized by the initial cell volume. (c) and (d) the TFI is normalized by the final cell volume. The TFI rises sharply during the pulse ((b) and (d)). After the pulse ends, it continues to grow until plateauing at around 1500 ms ((a) and (c)). The error bars in the plots represent standard error. The TFI is inversely proportional to the extracellular conductivity for all cases.
Figure 3.5: (a) The Total fluorescence intensity (TFI) extracted from Fig. 3.4(a), and normalized by the maximum achievable TFI from saponin treated cells. (b) The TFI extracted from Fig. 3.4(c), and normalized by the maximum achievable TFI from saponin treated cells. (■) Normalized TFI at the end of acquisition ($t = 7000$ ms). (▲) Normalized TFI close to the end of the pulse ($t = 95$ ms). (●) Difference between the TFI at the end of acquisition and the TFI at the end of the pulse. The dashed curves are theoretical predictions of electrophoresis mediated transport, calculated with the initial radii (a) and final radii (b), respectively.

is presumably the only available mechanism. The contribution from electrophoresis was consistently higher than that from diffusion for all cases. For comparison, the dashed lines are model predictions of electrophoresis mediated delivery from our earlier work [63, 64]. The details of the formula and further discussion are found in the Discussion section.

3.4 Discussion

The main objective of the current study is to analyze the mechanisms for molecular delivery via spatially and temporally resolved optical measurements. To this end, our results corroborate with the model prediction by two members of our research group (JL and HL) that electrophoresis of the charged ions can be the dominant mode of transport [63, 64].

In Figures 3.1 and 3.2, our data show consistently that the peak fluorescence intensity, which indirectly indicates the peak PI concentration, is inversely correlated with the extracellular conductivity. We argue that this behavior is mediated by field-amplified sample stacking (FASS), which we proposed in [63, 64]. Briefly, FASS is
induced by the presence of a non-uniform electric field, which arises when a potential difference is applied across regions of different conductivities. The Ohmic equation dictates that the field strength is higher in the lower conductivity region (the extracellular buffer) compared to that in the higher conductivity region (the cytoplasm) ([63] Fig. 5(b)). This difference in field strength leads to different electrophoretic velocities in the two regions ([63] Fig. 5(a)). The ions slow down upon entering the cell cytoplasm, where the electrophoretic velocity is lower, causing “stacking” in that region. The ratio of concentration enhancement is given by Eqn. (16) in [63], where the maximum ion concentration within the cell is inversely correlated with the extracellular conductivity, $\sigma_e$. This mechanism offers a viable interpretation of the trends observed in Figs. 3.1 and 3.2. However, note that the correlation in the experiments is not exactly reciprocal with respect to $\sigma_e$. This discrepancy may be attributed to the spatial convolution (in depth direction) of the signal emitted by the bound PI to determine peak fluorescence intensity, whereas the model prediction is for local free PI concentration in the absence of any binding reaction.

Figure 3.5 further demonstrates the agreement between the experiment and the model prediction. The dashed lines represent calculations according to Eqn. (12) in [64]:

$$c_{\text{tot}} = t_p c_e \omega F z E_0 \pi a^2 \left( \frac{3\sigma_i}{2\sigma_e + \sigma_i} \right)$$  \hspace{1cm} (3.1)

This equation is a compact formula to calculate the total molecules delivered into the cytoplasm ($c_{\text{tot}}$) via electrophoretic transport, and has been validated by full numerical simulations [64]. In this equation $t_p$ is the pulse length, $c_e$ is the extracellular concentration of the target molecule, $\omega$ is the mobility of ions, $F$ is Faraday’s constant, $z$ is the charge number, $E_0$ is the field strength, $a$ is the cell radius, and $\sigma_i$ is the cytoplasm electrical conductivity. For the present calculation, $t_p = 100$ ms, $c_e = 100$ $\mu$M, $\omega = 1.83 \times 10^{-13}$ $\text{m}^2 \text{V}^{-1} \text{s}^{-1}$, $F = 96,485 \frac{\text{C}}{\text{mol}}$, $E_0 = 0.8$ kV/cm, $z = +2$, and $\sigma_i = 0.5$ S/m. The extracellular conductivities, $\sigma_e$, are described in Table 3.1. The initial radii are used for the result in Fig. 3.5(a), and the final radii are used in Fig. 3.5(b). Finally, to compare with the experimental data, we normalized $c_{\text{tot}}$ by 25 amole, which is an
estimate of the total binding sites within the cell according to the measurements by Kennedy et al. (see Fig. 4 therein) [56]. When compared with the normalized TFI at \( t = 95 \text{ ms} \), closer agreement is found in Fig. 3.5 (a), whereas an over-prediction is observed in Fig. 3.5 (b). For both cases, the model captures the dependence of total delivery on the extracellular conductivity (the slope of the curves), which suggests that it provides a useful formula to estimate molecular delivery via electrophoresis. The results also suggest that the non-uniformity in the electrical field distribution plays a critical role in mediating molecular transport. Furthermore, because electrophoresis is the dominant mode of delivery in these cases, FASS related mechanisms also contribute significantly to the inverse correlation observed for total uptake.

After the pulse ceases at \( t = 100 \text{ ms} \), diffusion is presumably the only mechanism mediating transport. Its contribution is represented by the lowest curves in Fig. 3.5, where a peculiar inverse dependence on the extracellular conductivity is also observed. The cause of this dependence is unknown. In [63], the pore area density is predicted to have only a weak correlation with \( \sigma_e \) varying from 1000 \( \mu \text{S/cm} \) to 5000 \( \mu \text{S/cm} \). Pucihar et al. demonstrated that the percentage of permeabilized cells was not affected by the extracellular medium conductivity [85]. More studies, following both experimental and modeling approaches need to be performed to identify the underlying physical mechanisms. On the other hand, we remark that the post-pulsation fluorescence might include contributions from the further binding of PI molecules already delivered into the cytoplasm during the pulse, as suggested by Fig. 4 in [63].

Finally, the present work has made an interesting discovery that swelling depends monotonically on the extracellular conductivity (Fig. 3.3). Note that all our solutions, regardless of the conductivity were isotonic. If the swelling is indeed controlled by the osmolarity of the cell, then the results suggest that more osmolarity enhancing molecules (e.g., \( \text{Mg}^{2+}, \text{Cl}^- \), and sucrose) enter the cell at higher extracellular conductivities. We are currently performing model studies to correlate this data with a theoretical understanding.
3.5 Conclusion

In this work, we performed an extensive investigation on the transport mechanisms involved during electroporation-mediated molecular delivery. We used time- and space-resolved fluorescence microscopy to continuously monitor the uptake of PI into single 3T3 fibroblasts, using six different extracellular buffer electrical conductivities. During the pulse, both the peak intensity and the total fluorescence intensity (TFI) exhibited an inverse correlation with extracellular conductivity. This behavior is explained by field-amplified sample stacking (FASS), which is an electrokinetic mechanism arising from the presence of a non-uniform electric field.

The respective contributions from electrophoresis and diffusion have been quantified. The former is shown to be consistently higher than the latter for the experimental conditions considered. Furthermore, the results are compared with a compact model to predict electrophoresis-mediated transport. A good agreement is found between the two, which validates the formula as a convenient tool to estimate molecular delivery via electrophoresis. The experimental quantification and modeling tools developed in this work are important contributions toward designing optimized protocols for a wide range of applications utilizing electroporation.

We have also discovered that swelling depended monotonically on the extracellular conductivity, despite the fact that the buffer solutions were all isotonic. If the swelling is indeed controlled by the osmolarity of the cell, then the results suggest that more osmolarity enhancing molecules, other than PI, enter the cell at higher extracellular conductivities. We are currently performing model studies to correlate this data with a theoretical understanding.
Chapter 4

Double Pulse Electroporation-Mediated Molecular Delivery and Cell Viability

4.1 Introduction

In this chapter, the molecular delivery and cellular viability of electroporated 3T3 cells are optimized using two square electric pulses. The cells are permeabilized using a first pulse with relatively high electric field strength for a short period of time. At the end of the first pulse, a relatively longer second pulse is applied to manipulate both the degree of permeabilization and the electrophoretic transport. Fluorescein-dextran (FD) and 7-Aminoactinomycin D (7-AAD) were utilized to quantify the molecular delivery and viability, respectively. The fluorescence measurements were acquired using flow cytometry post-pulsation. The delivery and viability for the different second pulses are compared based on the electric field strength ($E_2$) and duration ($t_2$) of the second pulse.

The main objectives of the current work are to exploit the second pulse parameters and to define relationships for molecular delivery and cell viability based on physical principles. Subsequently, these relationships could be utilized to attain critical regimes for maximized delivery efficiency and viability.

Traditionally, a single electroporation pulse is utilized to permeabilize the cell membrane, while simultaneously driving exogenous molecules into the cell cytoplasm. An appreciable delivery can be achieved using high electric field strengths for tens of milliseconds [11, 16, 23, 27]. However, a large portion of the permeabilized cells do not survive post the electroporation shock. To enhance both delivery and viability different electroporation pulsing schemes were needed [84, 101, 103]. For instance, multiple hundred microns pulses with high field strengths were applied at a specific frequency to increase the permeability. Eventhough the delivery of small molecules ($MW < 4$
kDA) was efficient using these micron pulses, macromolecular delivery did not improve as these molecules require electrophoretic transport [91]. An alternative approach was to design double pulses: a short first pulse that have the ability to permeabilize the cell membrane and a long second pulse to drive molecules into the cytoplasm electrophoretically. The efficacy of double pulses was demonstrated by Sukharev et al. In their work, they showed that extending the second pulse duration enhanced DNA uptake [103]. Krassowska and co-authors confirmed the enhanced delivery of the double pulse electroporation approach through simulation and modeling efforts [98]. Moreover, in [98], the authors attributed the enhanced delivery efficiency to the coupled effects of the pore size dynamics and electrophoretic transport. Consequently, various studies utilized electroporation protocols with double pulses [11, 101, 103]. Unfortunately, the pulse parameters were empirically optimized and no correlations involving delivery and viability with respect to the pulsing parameters have been established.

In this work, we employed double pulses to enhance molecular delivery and viability. In addition, we utilized the results to identify critical regimes for maximized delivery and viability. The experimental parameters were chosen based on the experimental electroporation literature. The applied first pulse had a relatively strong field strength ($E_1 = 100,000$ V/m) and short duration ($t_1 = 0.001$ s) to permeabilize the cells. This pulse has been shown to promote significant permeabilization with moderate delivery and high viability [32, 58, 83, 84, 91, 98]. The field strength for the second pulse was varied from $E_2 = 10,000$-$100,000$ V/m and applied for longer durations raging $t_2 = 0.01$-$0.1$ s to promote electrophoretic delivery [84, 98, 101, 103, 123]. Using flow cytometry, a statistically significant number of cells was analyzed quantitatively to assess both delivery and viability [11, 82, 83]. The delivery of FD molecules into 3T3 mouse fibroblast cells was quantified. Due to FD size (MW $\sim 10,000$ Da), the effects of passive diffusion on delivery were limited, and only the electrophoretic transport controlled the final delivery of FD into the cells [82, 91, 107, 123]. Cellular viability was assayed using 7-Aminoactinomycin D (7-AAD) due to its optical properties. The combination of FD and 7-AAD required minimal compensation. Moreover, a low conductivity electroporation buffer was utilized to minimize joule heating effects. This work is the first
step towards optimizing delivery and viability based on physical insights as opposed to empirically derived protocols.

4.2 Materials and Methods

4.2.1 Cell Culture

NIH 3T3 mouse fibroblasts were plated in T75 flasks and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with Fetal Bovine Serum (FBS) (10% v/v), Penicillin-Streptomycin (1% v/v), and L-Glutamine (1% v/v) (Sigma, St. Louis, MO) at 37°C and 5% CO₂. Prior to each experiment, cells were harvested at 70-80% confluency using 0.5% trypsin (Sigma) and washed once with culture media. The cell suspension is spun for 2 min at 460×g (2000 rpm, Allegra X-21, Beckman Coulter) and washed twice with the electroporation buffer containing 0.5 mM MgCl₂, 200 mM sucrose, and 10 mM HEPES (pH 7.4). The electrical conductivity and osmolality of the electroporation buffer were 100 µS/cm and 310 mOsm/kg, respectively.

4.2.2 Electroporation Protocol

Approximately 3×10⁶ cells/mL were suspended in electroporation buffer containing 100 µM of Fluorescein-dextran (FD) (Life Technologies) and incubated on ice for 5 minutes before pulsation. FD has an average MW ~ 10,000 Da, diffusivity in water $D_{FD-10}=9.8 \times 10^{-7}\text{cm}^2/\text{s}$, net charge $z = -0.1$, and fluorescence properties of fluorescein (ex: 494 nm, em: 524 nm). The electroporation experiments were conducted in room temperature (20°C) under a sterile hood. A volume of 90 µL of cell suspension is placed into an electroporation cuvettes with 1 mm gap (VWR). Various combinations of double pulses were applied using a custom-built electroporator that can deliver calibrated and controlled square pulses (Fig. 4.1). The first pulse was programmed at $E_1 = 100,000 \text{ V/m}$ and $t_1 = 0.001 \text{ s}$ in strength and duration at all times, respectively. Ten electric fields were used for the second pulse ($E_2$), namely 10,000 V/m, 20,000 V/m, 25,000 V/m, 30,000 V/m, 35,000 V/m, 40,000 V/m, 50,000 V/m, 60,000 V/m, 80,000 V/m and 100,000 V/m. The duration of the second pulse ($t_2$) was changed
Figure 4.1: Schematic depicting an exemplary double pulse utilized in the delivery of Fluorescein-dextran into 3T3 mouse fibroblasts. The first pulse was 100,000 V/m in strength and 0.001 s duration, and the second pulse was 30,000 V/m in strength and 0.01 s duration.

from 0.01 s to 0.1 s. Post pulsation, complete media was added to the electroporated cells. The cell suspension was incubated for a recovery period of 15 minutes. To minimize FD fluorescence background, cells were washed twice with phosphate buffer saline without magnesium and calcium (PBS) (Sigma). The cells were then resuspended in PBS containing 2 μM of 7-Aminoactinomycin D (7-AAD) (MW = 1270.43 Da) (Life Technologies). Upon binding to DNA/RNA, 7-AAD emits a fluorescent signal (ex: 488 nm, em: 650 nm) to discriminate between viable and dead cells. For the present experimental conditions, the temperature changes due to joule heating did not exceed 2.5°K for the highest second pulse conditions, the corresponding calculations are shown in Appendix B.

4.2.3 Flow Cytometry Data Acquisition

Beckman Coulter FC500 analyzer flow cytometer (Beckman Coulter) and CXP Analysis software were utilized to perform measurements of non-electroporated and electroporated cells. The data was acquired at a medium flow rate (~ 30 μL/min). The samples were excited with 488 nm Argon laser. Forward scatter (FSC) and side scatter (SSC) measurement were used to distinguish between cells and debris based on cell size and granularity, respectively. After the cell population is gated based on FSC and SSC of
the control condition (non-pulsed cells), 10,000 events were acquired within the pre-selected gate for each condition. Channel 1 (FL1) in the FACS machine contained a 525 nm long pass filter. FL1 was utilized to measure the level of intracellular FD fluorescence signal in each cell. Channel 4 (FL4) has a 675 nm long pass filter, and it was used to measure the level of 7-AAD in each cell and to separate dead cells from viable ones. Prior to each experiment, spectral compensation was performed due to the overlap of the FD and 7-AAD signals. Compensation was conducted with three cell populations: regular cells that did not contain any fluorescence, cells loaded with 7-AAD only, and cells loaded with FD only. The data from all channels was acquired in a logarithmic scale.

4.2.4 Flow Cytometry Data Analysis

Cell population is separated from debris using the FSC and SSC plot as demonstrated with the circular gate in Fig. 4.2(a). Subsequently, the gated cell population is expanded into a dot plot with FL1 (x-axis) and FL4 (y-axis) to distinguish live/dead cells, and the degree of delivery (FD) in live cell (Fig. 4.2(b), (c) and (d)). The limits for the quadrants in the scatter plots were pre-selected based on the compensation analysis discussed in section 4.2.3. Therefore, cells in each quadrant represent the following cell populations:

- Q1: Dead cells that contain 7-AAD only
- Q2: Dead cells that contain both 7-AAd and FD
- Q3: Live Cells that contain FD only
- Q4: Live cells that contain no fluorescence

Figure 4.2(b), (c) and (d) represent dot plots of cells electroporated with different pulse conditions. Fig. 4.2(b) represents control condition with cells that were not electroporated. Fig. 4.2(c) and (d) represent cells electroporated with a single, and a double pulse, respectively. The cells above the horizontal line are dead cells containing 7-AAD (Q1 and Q2 quadrants). Despite that the fluorescence detector of FL-4 channel
Figure 4.2: Flow cytometry analysis of non-electroporated and electroporated cells using dot plots. The cells in (a) represent control cells that were not pulsed. These cells are plotted with respect to forward scatter (x-axis) and side scatter (y-axis). The cell population in (a) is separated from debris using a circular gate. In (b), (c), and (d), the x-axis represents the level of the Fluorescein-dextran (FD) signal (delivery) in cells, and the y-axis represents the level of the 7-Aminoactinomycin D (7-AAD) signal (viability) in cells. Furthermore, the horizontal line separates live cells from dead cells, and the vertical line separates cells with and without FD. Cells in (b) were the non-pulsed cells from (a). The cells in (c) were electroporated with a single pulse ($E_1 = 100,000 \, \text{V/m}, t_1 = 0.001 \, \text{s}$). The cells in (d) were electroporated with a double pulse ($E_1 = 100,000 \, \text{V/m}, t_1 = 0.001 \, \text{s}, E_2 = 30,000 \, \text{V/m}, t_2 = 0.1 \, \text{s}$).
was not saturated, the majority of dead cells aggregated at a specific fluorescence level for all the experimental conditions indicating saturation of the binding spots for 7-AAD in dead cells. The percentage of viable cells was calculated by counting the number of viable cells present in the Q3 and Q4 quadrants and normalized by the total number of live cells from the control non-pulsed condition (Fig. 4.2(b)). Only viabilities equal to or higher than 50% were analyzed henceforth.

The vertical line in Fig. 4.2(b), (c) and (d) separated cells with and without FD (Q3 and Q4 quadrants). The cells from Fig. 4.3(b) were not pulsed, therefore, these cells did not contain any FD and all the cells resided in the Q4 quadrant. The application of an electroporation pulse ($E_1 = 100,000$ V/m, $t_1 = 0.001$ s) shifted the cell population partially into the Q3 quadrant indicating FD delivery into cells (Fig. 4.3(c)). The addition of a second electric pulse ($E_2 = 30,000$ V/m, $t_2 = 0.1$ s) to the single pulse pushed the cell population further in the Q3 quadrant (Fig. 4.3(d)) indicating enhanced FD delivery. To assess the FD delivery efficiency, the histograms of the cells in Q3 were plotted in Fig. 4.3. The median value of the distribution curves from Fig. 4.3 were calculated. The delivery efficiency is obtained by subtracting and normalizing the median fluorescence signal of each condition by that of the first pulse. All mathematical calculations were performed with MATLAB (The Mathworks, Inc., Natick, MA).

4.3 Results

The effects of the second pulse duration ($t_2$) and strength ($E_2$) on the delivery of Fluorescein-dextran (FD) into 3T3 mouse fibroblast cells are compared in Fig. 4.4. The intracellular fluorescence signal of the delivered FD with double pulses was subtracted and normalized by that of the first pulse signal ($E_1 = 100,000$ V/m and $t_1 = 0.001$ s). Fig. 4.4(a) demonstrates that the normalized fluorescence signal increases linearly with increasing the second pulse duration ($t_2$) when the field strength is held constant. Furthermore, the second pulse enhancing effects become noticeable only when the second pulse field strength is higher than $E_2 = 25,000$ V/m. Fig. 4.4(b) shows that the normalized fluorescence signal changes as a sigmoid with respect to $E_2$ at constant $t_2$. The fluorescence signal is very low and comparable to that of the first pulse when
Figure 4.3: The histograms demonstrate the fluorescence signal of delivered Fluorescein-dextran (FD) into live 3T3 mouse fibroblast cells for different electroporation conditions. The cell populations of each distribution are obtained from the Q3 quadrant in Fig. 4.2(b), (c) and (d). The solid red line represents non-pulsed cells. This population has a very small peak due to the low number of cells. The blue dashed line represents cells electroporated with a single pulse ($E_1 = 100,000$ V/m, $t_1 = 0.001$ s). The dotted orange line represents cells electroporated with a double pulse ($E_1 = 100,000$ V/m, $t_1 = 0.001$ s, $E_2 = 30,000$ V/m, $t_2 = 0.1$ s). Applying an electroporation pulse increased the number of cells containing FD. The addition of a second pulse enhanced the delivery by shifting the distribution further to the right.
Figure 4.4: Normalized fluorescence signal of intracellular Flourescein-dextran is plotted with respect to the second pulse duration ($t_2$) (a) and field strength ($E_2$) (b). The double pulses signal was subtracted and normalized from that of the first pulse. The first pulse was always $E_1 = 100,000$ V/m and $t_1 = 0.001$s. The second pulses field strengths ranged from $E_2 = 10,000$ V/m to $E_2 = 100,000$ V/m with durations from $t_2 = 0.01$ s to $t_2 = 0.1$ s. The fluorescence signal is linearly proportional to $t_2$ and changes as a sigmoid with $E_2$.

$10,000$ V/m $\leq E_2 \leq 20,000$ V/m. The fluorescence signal rises when $E_2$ is increased to 25,000 V/m and continues to increase slowly until $E_2$ reaches 40,000 V/m. From $E_2 = 40,000$ V/m to $E_2 = 60,000$ V/m the signal rises sharply before it plateaus. Furthermore, the plateau values increase with increasing $t_2$ (Fig. 4.4(b)).

The normalized fluorescence curves in Fig. 4.4(a) are fitted with the following linear equation:

$$N.F. = \tau_f(E_2) \times t_2$$  \hspace{1cm} (4.1)

where $N.F.$ represent the normalized fluorescence signal, and $\tau_f$ is the delivery per unit time. The $\tau_f$ values are plotted with respect to $E_2$ in Fig. 4.5. The error bars represent the 95% confidence interval of the linear curve fits in Fig. 4.4(a). In Fig. 4.5, the delivery per unit time changes also as a sigmoid. The $\tau_f$ starts at $E_2 = 25,000$ V/m, and increases slowly until it reaches $E_2 = 40,000$ V/m. The delivery per unit time rises sharply until it reaches $E_2 = 60,000$ V/m and plateaus. The sigmoid is fitted with this equation:

$$\frac{dN.F.}{dt_2} = \frac{1310}{1 + e^{-\frac{50,000 - E_2}{5156}}}$$  \hspace{1cm} (4.2)
Figure 4.5: The delivery per unit time ($\tau_f$) is plotted with respect to $E_2$ and fitted with a sigmoid curve (Eqn. (4.2)) as shown by the dashed line. The error bars represent the 95% confidence interval for the linear curve fitting in Fig. 4.4. The coefficient of determination $R^2 = 0.97$.

Furthermore, integrating Eqn. (4.2) with respect to $t_2$ results in an equation of the normalized fluorescence intensity with respect to $E_2$ and $t_2$:

$$N.F. = \frac{1310}{1 + e^{50,000 - E_2 \times 5156}} \times t_2 \quad (4.3)$$

In Figure 4.6, the viability percentage of cells electroporated with double pulses is plotted with respect to second pulse duration ($t_2$) and field strength ($E_2$). The maximum viability was $\sim 97\%$ and corresponds to cells electroporated with the first pulse only. The viability decreases linearly with increasing ($t_2$) and quadratically with increasing the field strength ($E_2$) as demonstrated in Fig. 4.6(a) and (b), respectively. More importantly, the viability scales linearly with respect to the electrical energy of the second pulse ($E_2^2 t_2$) as shown in Fig. 4.7. The data in Fig. 4.7 is fitted with this equation:

$$V(\%) = 97.3 - 1.49 \times 10^{-7} E_2^2 t_2 \quad (4.4)$$

where $V$ represent the percentage of viable cells.

The equations of delivery (Eqn. (4.3)) and viability (Eqn. (4.4)) with respect to the
Figure 4.6: The viability percentage of cells electroporated with double pulses is plotted with respect to the second pulse length (a) and field strength (b). The double pulses signal was subtracted and normalized from that of the first pulse. The first pulse was always $E_1 = 100,000 \text{ V/m}$ and $t_1 = 0.001\text{s}$. The second pulses field strengths ranged from $E_2 = 10,000 \text{ V/m}$ to $E_2 = 100,000 \text{ V/m}$ with durations from $t_2 = 0.01 \text{s}$ to $t_2 = 0.1 \text{s}$. The viability percentage is linearly proportional to $t_2$ and quadratically proportional with $E_2$.

Figure 4.7: Scaling law of viability percentage with respect to the electrical energy ($E_2^2 t_2$). Viability decreases with increasing electrical energy.
Figure 4.8: Fluorescence intensity is calculated from Eqn. (4.5) for viabilities ranging between 50% and 95%. The fluorescence intensity is plotted with respect to $E_2$ in (a) for constant viability lines. The fluorescence intensity increases until it reaches a local maximum at $E_2 = 58,000$ V/m, then decreases. The maximum fluorescence intensity at the critical point ($E_2 = 58,000$ V/m) is plotted with respect to viability percent in (b). An inverse relation between fluorescence signal and viability percent is observed.

Second pulse parameters ($E_2$ and $t_2$) are utilized to calculate the fluorescence intensity at constant viabilities. More importantly, a critical regime where both delivery and viability are maximized was achieved from solving this system of equations. Eqn. (4.4) is solved for $t_2$ at known viabilities (50%-95%) and plugged into Eqn. (4.3) to obtain the following relationship between the normalized fluorescence signal ($N.F.$) and $E_2$:

$$N.F. = \frac{1310}{1 + e^{\frac{50,000 - E_2}{5156}}} \times \frac{(97.3 - V)}{1.45 \times 10^{-7} \times E_2^2}$$

(4.5)

Fig. 4.8(a) shows the calculated $N.F.$ values plotted with respect to $E_2$. The fluorescence increases until it reaches a maxima at $E_2 = 58,000$ V/m, then it signal decreases until it reaches $E_2 = 100,000$ V/m. Furthermore, the fluorescence maxima values at $E_2 = 58,000$ V/m are plotted with respect to the viability percentage in Fig. 4.8(b) and demonstrate an inverse correlation between the maximum achievable fluorescence intensity and viability.
Figure 4.9: The transmembrane potential ($V_m$) and electrophoretic velocity ($U_e$) plotted with respect to the second pulse electric field strength ($E_2$). The transmembrane potential in (a) was calculated with Eqn. (1.1). $V_m$ changes linearly with respect to the field strength at constant cell radius. Different dashed lines and symbols represent the change in the transmembrane potential with respect to the cell radius. The electrophoretic velocity of Fluorescein-dextran (FD) in (b) was calculated by $U_e = \mu_e E_2$, where $\mu_e$ denotes the electrical mobility of fluorescein dextran. The electrophoretic velocity of FD is linear with respect to $E_2$.

4.4 Discussion

The main focus of the current study is to enhance electroporation protocols by applying double pulses to improve molecular delivery and maintain cell viability and functionality. Both delivery and viability during electroporation are controlled by the electric field strength and duration. The field strength is paramount in determining the transmembrane potential (TMP) (Fig. 4.9(a)), and the electrophoretic mobility of the delivered molecules (Fig. 4.9(b)). Furthermore, the TMP is critical in determining the permeabilized area on the membrane, and the pore size [84, 98, 101, 103, 123]. The changes in the normalized fluorescence signal in Fig. 4.4 demonstrate that delivery may be controlled and enhanced by mechanisms involving electrophoretic transport and permeabilization. For instance, increasing the field strength increases the electrophoretic mobility and the permeabilized area leading to an increase in the amount uptaken by the cell (Fig. 4.4(b)). On the other hand, increasing the pulse duration increased only the electrophoretic transport resulting on a linear increase in fluorescence signal (Fig. 4.4(a)).

The coupled effects of electrophoresis and permeabilization may lead to the different
delivery regimes observed in Fig. 4.4. During the first pulse, large pores were created due to the high TMP (∼1 V) as shown in Fig. 4.4(a) [98]. After the first pulse ended at 0.001 s, the electric field strength changed to $E_2$ and the transmembrane potential at the cell membrane either remained the same or dropped depending on the value of $E_2$ (Fig. 4.9(a)). In the first regime ($E_2 \leq 20,000$ V/m), the delivery was weak mainly because the pore size ($r_{\text{pore}}$) shrank to a radius smaller than the size of the coiled FD polymer chain ($r_{FD} = 2.3$ nm). Since $r_{pore} < r_{FD}$, the delivery of the coiled FD via electrophoresis was restricted, therefore transport would have happened only through entropic trapping phenomenon represented by the translocation of unfolded FD [122]. As the field was raised above 20,000 V/m, the delivery increased slowly until $E_2$ reached 40,000 V/m. This slow increase in delivery indicates a transition region where $r_{pore} \simeq r_{FD}$. In this transition region, coiled FD molecules were transported with electrophoresis through pores with $r_{pore} = r_{FD}$ and entropic trapping for pores $r_{pore} < r_{FD}$ leading to an improved delivery overall. When 40,000 V/m $\leq E_2 \leq 60,000$ V/m, the delivery rose rapidly due to presence of pores larger than the size of FD ($r_{pore} > r_{FD}$) and large magnitudes of the electrophoretic velocities. The coupled effects of large pores ($r_{pore} > r_{FD}$) and high electrophoretic velocities enhanced the delivery tremendously reaching efficiencies up to 94 times better than that of the first pulse. After $E_2 = 60,000$ V/m, the delivery reached a plateau. Additionally, when $E_2 = 80,000$ V/m and 100,000 V/m, and specifically when the field was applied longer than $t_2 = 0.04$ s, the number of viable cells began to plummet below 50% indicating the inability of cells to reseal these large pores (data not shown). The inability of cells to reseal large pores could have led to leakage of FD post pulsation which subsequently could have inhibited further delivery enhancement.

As opposed to delivery, increasing the second pulse parameters led to a decrease in viability as shown in Fig. 4.6. Increasing the duration ($t_2$) of the applied electric field decreased viability linearly for constant electric fields (Fig. 4.6(a)). On the other hand, the viability decreased quadratically with increasing the field strength Fig. 4.6(b)). Interestingly, the viability scaled linearly with the electrical energy of the second pulse ($E_2^2 t_2$) as shown in Fig. 4.7. This linear relationship manifests that cellular viability
decreases with increasing the energy in the system. The permeabilized area, pore size, and electrophoretic transport increased with increasing the second pulse parameters which may have caused a significant loss of ions, proteins and other molecules necessary for cellular homeostasis with increasing $E_2$ and $t_2$. In addition, the long pulses alter the pH and chemical structure of the buffer molecules which induces toxic effects on cells that affects their viability negatively [84, 95].

The delivery and viability were optimized with respect to the electric field parameters (Fig. 4.8). The system of equations of delivery (Eqn. (4.3)) and viability (Eqn. (4.4)) were solved to obtain an equation for FD fluorescence (delivery) as a function of viability and the second pulse parameters (Eqn. (4.5)). Using Eqn. (4.5), the delivery with respect to $E_2$ was solved for viabilities ranging from 50% to 90% (Fig. 4.8(a)). The calculated fluorescence intensity for all viability curves increased with increasing the field strength until they reached a local maxima at $E_2 = 58,000$ V/m, then the fluorescence decreased. Moreover, an inverse relationship between delivery and viability was observed (Fig. 4.8(b)). These results demonstrate that the electric field parameters need to be optimized systematically to achieve better delivery and viability.

### 4.5 Conclusion

In this work, we performed electroporation experiments to enhance delivery and cellular viability. We split the electric pulse into two stages; a short, strong pulse to permeabilize the membrane with minimal cell damage, and a second pulse with long duration to extend the electrophoretic transport. The different delivery regimes demonstrate that the second electric pulse parameters might have affected the permeabilized area and pore size. Above a threshold electric field, delivery increased linearly with increasing the second pulse length. Below this electric field threshold, the transmembrane potential was below the critical permeabilization requirement, hence, the electrophoretic transport was hampered. However, above the critical threshold, the pore size expanded, allowing electrophoretic transport. When the field strength was increased, the combined effects of permeability and electrophoretic transport resulted in an enhanced delivery. Moreover, the three delivery regimes were well fitted with a sigmoid function. Viability
decreased with increasing the second pulse parameters. More importantly, viability scaled linearly with the electrical energy of the system. The delivery and viability were optimized systematically by solving a system of equations for delivery and viability. The results demonstrated a critical regime where both delivery and viability are maximized at an approximate second pulse field strength of 58,000 V/m.
Chapter 5

Conclusion

In this thesis, we have designed and implemented experiments to study the electrodeformation of giant unilamellar vesicles, to investigate the complex transport mechanisms, and to enhance molecular uptake and cellular viability associated with electroporation. Accordingly, the contributions of this dissertation are as follow:

1. We have systematically characterized large electrodeformation of giant lipid vesicles with direct optical observation. The combination of strong DC electric fields and high intra-to-extra-vesicular conductivity ratios induced high aspect ratios reaching 13 in some cases. The experimental and theoretical analysis provide physical insight on the behavior of membranes in a complex domain where strong, non-linear deformation is coupled with a significant poration of the membrane. This work has been summarized in a publication in *Physical Review E* journal [93].

2. We have quantitatively analyzed the mechanisms for molecular delivery via spatially and temporally resolved optical measurements. We found that electrophoresis of the charged ions can substantially enhance delivery. Furthermore, we found that field-amplified sample stacking (FASS), an electokinetic mechanism resulting from the presence of a gradient of electrophoretic velocity, plays a critical role in controlling the achievable molecular concentration within the cell. The results are compared with a compact model to conveniently predict electrophoresis-mediated transport. The experimental quantification and modeling tools developed in this work are important contributions toward designing electroporation protocols based on physical fundamentals. This work has been summarized in a publication in *Biochimica et Biophysica Acta (BBA) - Biomembranes* [94].
3. Extending from the task above, we have improved molecular delivery while maintaining significant cell viability by using a double pulse scheme. The electroporation pulse was split into two components: one for permeabilizing the cell membrane and one for electrokinetically-mediating transport. An inverse relation between delivery and viability is mediated by their strong dependence on the applied electric field parameters. Furthermore, a critical regime, where delivery and viability are maximized, is achieved based on the optimization of field strength and pulse duration. This contribution is a significant advance toward the development of protocols based on physical principles as opposed to the current empirical protocols.

We hope that the framework of this dissertation offers new physical insight that can help with understanding, developing, and optimizing current and new applications of vesicle deformation and electroporation-mediated molecular delivery.
Appendix A

Coefficients used in Eqn. (2.20)

The coefficients $K_0$, $Q_\mu$, $Q_F$ appearing in Eqn. (2.20) are defined with the following expressions:

$$K_0 = -\frac{\pi \rho_m a_0^4 \tan(\Theta)}{4 \sin^2(\Theta) \phi(\Theta) \sin(\Theta) + \Theta \pi} \left[ -\Theta^3(1 + 8 \cos^4(\Theta)) + \Theta^2 \cos(\Theta) \sin(\Theta)(6 \cos^2(\Theta) - 1) 
+ \Theta \cos^2(\Theta) \sin^2(\Theta)(1 + 4 \cos^2(\Theta)) + \cos^3(\Theta) \sin^3(\Theta)(1 - 2 \cos^2(\Theta)) \right],$$

(A.1)

$$Q_\mu = -\frac{2 \mu_0 a_0^4 \pi}{\cos(\Theta) \sin(\Theta) \phi(\Theta) \sin(\Theta) + \Theta \pi} \left[ \cos(\Theta) \sin(\Theta)(2 \cos^2(\Theta) + 1) - \Theta(4 \cos^2(\Theta) - 1) \right] 
- \frac{4 \mu_0 a_0^4 \pi}{\sin(\Theta) \phi(\Theta) \sin(\Theta) + \Theta \pi} \times [2 \Theta^3 \cos(\Theta)(2 \cos^2(\Theta) + 1) - \Theta^2 \sin(\Theta) 
+ 2 \Theta \cos(\Theta)(\cos^4(\Theta) - 1) - \cos^2(\Theta) \sin^3(\Theta)] \right],$$

(A.2)

$$Q_F = \pi \varepsilon E^2 B^2 a_0^3 (\gamma^2 - 1) \{ -\frac{4}{3} \sqrt{\frac{2 \cos^4(\Theta) \sin(\Theta)}{\phi(\Theta) \sin(\Theta) + \Theta \pi}} - 2 \sqrt{\frac{2 \cos^4(\Theta)}{\sin^5(\Theta) \phi(\Theta) \sin(\Theta) + \Theta \pi}} \}
\times \left[ \sin(\Theta) - \text{arctanh}(\sin(\Theta)) \right] \phi(\Theta)(2 \cos^2(\Theta) + 1) + \cos(\Theta) \sin(\Theta) \}. $$

(A.3)
Appendix B

Temperature change during double pulse electroporation experiments

Temperature change ($\Delta T$) during pulse application was calculated using the simplified joule heating equation:

$$
\Delta T = \frac{\sigma_e}{\rho c_p} (E_1^2 t_1 + E_2^2 t_2)
$$

(B.1)

where $\sigma_e$ is the buffer electrical conductivity, $\rho$ is the density of water, $c_p$ is the specific heat capacity of water, $E_1$ is the electric field strength of the first pulse, $t_1$ is the duration of the first pulse, $E_2$ is the electric field strength of the second pulse, $t_2$ is the duration of the second pulse. For the present experimental conditions, the temperature change due to joule heating is plotted as a function of $t_2$ (Fig. B.1(a)) and $E_2$ (Fig. B.1(b)). The temperature change for all conditions was less than 2.5$^\circ$K.

![Figure B.1: Temperature change due to joule heating in double pulse electroporation experiments. Temperature rises linearly with time (a) and quadratically with field strength (b).](image)
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