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The Molecular Selectivity of Non-Thermal Irreversible Electroporation
and Tissue Regeneration *In Vivo*

By

Mary Alice Phillips

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor in Philosophy

in

Engineering – Mechanical Engineering

in the

Graduate Division

Of the

University of California, Berkeley

Committee in charge:

Professor Boris Rubinsky, Chair

Professor Ralph Greif

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ABSTRACT

The Molecular Selectivity of Non-Thermal Irreversible Electroporation

and Tissue Regeneration *In Vivo*

by

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Non-thermal irreversible electroporation (NTIRE) is new minimally-invasive surgical technique for tissue ablation that utilizes molecular selectivity to ablate tissue tumors. Short, microsecond electrical pulses are applied to the tissue, selectively targeting the cell membrane, causing pores to form within the membrane and leading to cell death. This tissue ablation technique has potential for a variety of medical applications, and has shown great promise as a method for treating cancer tumors. NTIRE has many promising attributes as a treatment modality, such as the preservation of tissue scaffolding and the blood vessels. Very little work, however, has been done in examining how the molecular selectivity of NTIRE affects tissue regeneration.

This work examines how tissues regenerate and recover after NTIRE, with a focus on those critical tissues that are particularly susceptible to collateral damage from treating an adjacent tumor. Two important tissues are examined: the artery and the small intestine. The artery may be embedded within a tumor. Although complete tumor ablation is desired, it is important that the artery can recover quickly in order to aid in overall tissue regeneration at the treated site. It is also important to understand how the molecular selectivity of NTIRE affects the regeneration of the small intestine, especially for the application of abdominal cancer treatment. Damage to the small intestine is often the limiting factor in other types of cancer treatments such as localized radiation therapy, causing pain and discomfort and even resulting in stopping the treatment early. Understanding how the small intestine recovers after NTIRE is essential in developing this technology for treating abdominal cancers such as pancreatic cancer.

Finite element models were utilized to design electrical parameters for both the artery and the small intestine that would cause irreversible electroporation to occur within the tissue while avoiding thermal damage due to Joule heating effects. These electrical parameters were then applied *in vivo*. Electrical parameters chosen to apply to the artery were an electric field of 1750 V/cm, 90 pulses of a pulse length of 100 μ s, and a frequency of either 1 or 4 Hz. The chosen small intestine electroporation protocol consisted of 2000 V/cm, 50 pulses of 70 μ s each, and a frequency of 4 Hz. Additional finite element analysis was used to examine the effect of the heterogeneity of tissues such as the small intestine, indicating that changes in electrical

conductivity from layer to layer is an important factor that should be accounted for in clinical treatment planning, and future work should include quantifying these electrical conductivity values.

By applying NTIRE to the rat carotid artery, the recovery of the artery over the week following treatment was observed. It was demonstrated that the electroporation protocol preserved the native tissue extracellular matrix. Three days after NTIRE treatment, the ablated cells had been naturally removed from the tissue, leaving a decellularized construct. By one week after electroporation, new endothelial cells were seen lining the artery lumen. This endothelial layer indicates that normal recellularization is taking place and that the artery is beginning to recover within 7 days of treatment.

In a similar fashion, NTIRE was applied to the rat small intestine *in vivo*, and the recovery of the small intestine was observed during one week post-treatment. The electrical parameters used were shown to be strong enough to initially cause complete cellular destruction. The extracellular matrix, however, appeared undamaged, and the structure of the small intestine remained intact. The intestine showed signs of recovery, developing an epithelial layer at 3 days post-treatment and regenerating mucosa, submucosa, and muscular layers within a week. These results suggest that the small intestine is only temporarily affected by NTIRE, indicating that this procedure can be utilized for abdominal cancer treatment while minimizing collateral damage to adjacent tissues.

In addition to examining the recovery of the artery for cancer treatment applications, the potential use of NTIRE to develop a decellularized arterial scaffold was also investigated. The tissue scaffold is a key component for tissue engineering, and the extracellular matrix is nature's ideal scaffold material. Two different methods for applying NTIRE to the artery were compared; the results obtained when plate electrodes were applied across the rat carotid artery were compared to the case when endovascular electrodes were applied to the rabbit iliac artery in a minimally invasive fashion. Both methods were shown to preserve the native extracellular matrix and produce a scaffold that is functional and facilitates recellularization. At 3 days post NTIRE, the immune system had decellularized the electroporated tissue, leaving behind a functional scaffold. The endothelial regrowth at 7 days after treatment indicates that the extracellular matrix still maintained its important components to support cell growth. In addition, this endothelial layer shows promise for the tissue scaffold, helping it to avoid issues such as thrombogenicity that many small diameter scaffolds face.

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NOMENCLATURE

γ	edge energy at the pore walls
ϵ_w	permittivity of pure water
ϵ_m	permittivity of the lipid interior
θ	angle between the electric field and the point of interest on the cell membrane
ρ	density
σ	electrical conductivity
σ_{cm}	circumferential muscle layer electrical conductivity
σ_{ll}	electrical conductivity of muscle fibers parallel to electric field
σ_t	electrical conductivity of the inner layers of the small intestine
σ_T	electrical conductivity of muscle fibers perpendicular to electric field
τ	time constant of the cell membrane
ϕ	electric potential
ω	perfusion rate
Γ	effective tension of the membrane
ΔW_p	pore formation energy
ΔE	energy barrier height
Ω	thermal damage parameter
A	rate constant of cell membrane damage accumulation
$C(0)$	concentration of undamaged molecules at time zero
$C(t_2)$	concentration of undamaged molecules at time t_2
C_{LW}	change in specific capacitance
C_o	capacitance per unit area of pore-free membrane thickness
C_p	heat capacity
E	applied electric field

E_a	activation energy
E_m	transmembrane electric field
f	frequency
f_c	cell shape factor
F_d	fraction of damaged molecules
g	relative electric permeability of the membrane
h	cell membrane thickness
h_{conv}	convection coefficient
k	thermal conductivity
K	rate at which tissue becomes thermally damaged
L	characteristic length of the cell in the longer direction
N	number of pulses
q	basal metabolic heat generation
q_{JH}	heat generation per unit volume
r	cell radius
r_c	critical pore radius
r_p	pore radius
R	ideal gas constant
t	time
t_l	pulse length
T	temperature
T_∞	air temperature
T_a	tissue temperature
T_o	initial temperature
U	transmembrane potential
V_o	applied voltage

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION TO MOTIVATION

This thesis focuses on the effect of irreversible electroporation on biological tissues with time. Currently irreversible electroporation is being developed as a method for ablating cancer tumors. Non-thermal irreversible electroporation (NTIRE) has great potential as a new tissue ablation modality and has reached clinical trials for some types of cancer treatment. Thus, it is important to understand how tissues near or embedded within the tumor will respond and recover after being electroporated. Additional research into how NTIRE affects tissue recovery is essential in order to further expand the use of NTIRE to encompass a broader range of tissues that can utilize its cancer ablation effects. While long term studies show evidence that using NTIRE as an ablation modality spares tissue scaffolds and blood vessel structures, thus far there has been no systematic study on how NTIRE affects critical tissues such as the small intestine or the process of tissue regeneration. This knowledge will also serve to benefit the general field of irreversible electroporation as it is further developed for other medical purposes. Here, the artery and the small intestine are examined as two clinically relevant tissues. In addition, the effect of irreversible electroporation on the artery is examined in the context of developing a decellularized tissue scaffold for the development of a tissue engineered arterial graft. Here, a background of electroporation is given in order to further understand how to harness this technology, and a background of irreversible electroporation for cancer treatment as well as the development of tissue scaffolds is also provided, lending the base upon which this thesis work was built.

1.2 ELECTROPORATION

1.2.1 Introduction of Irreversible Electroporation

Non-thermal irreversible electroporation (NTIRE) is a new minimally invasive surgical technique that was originally conceived from theoretical considerations with the capability of selectively targeting cell membranes to treat biological tissues [Davalos *et al*, 2005]. Rather than using drug induced chemical selectivity, NTIRE is based on fundamental biophysical principles. The cell ablation technique used in this thesis deals with a bioelectric and a biothermal phenomenon. The bioelectric phenomenon is characterized by the permeabilization of the cell membrane's lipid bilayer through the application of very brief (nanosecond to millisecond), high field (in the range of MV/m) electric pulses across the cell [Weaver and Chizmadzhev, 1996; Weaver, 2000; Chen *et al*, 2006]. This biophysical phenomenon has been observed for centuries [Nollet, 1754] and studied intensively since the mid 1900s e.g. [Sale and Hamilton, 1967]. Several different names have been used in literature to describe this phenomenon; electropermeabilization is used to describe the physical effect of the pulses on the cell membrane [Stopper *et al*, 1985], and electroporation describes the hypothetical pores that form [Neumann *et al*, 1982]. The effects of electroporation depend on the magnitude and duration of the pulsed electric field as well as other factors such as cell size and shape and number of electrical pulses

applied. The electric field magnitude triggers pore formation [Teissie and Rols, 1993], whereas the pulse length influences the pore expansion process [Gabriel and Teissie, 1997].

The family of electrical pulses that cause electroporation are divided into two types; in reversible electroporation, the cells survive the permeabilization process, and irreversible electroporation results in cell death due to the lipid bilayer destabilization and permeabilization [Weaver and Chizmadzhev, 1996; Weaver, 2000; Chen *et al*, 2006]. Physical principles indicate that the energy dissipation of high electric fields such as those involved in electroporation can lead to an increase in tissue temperature due to Joule heating [Chang and Nguyen, 2004]. Indeed these thermal effects have been used clinically with such applications as radiofrequency, microwave, laser, high frequency ultrasound, and even conventional electric heating ablation [Davalos, 2005]. Such elevated temperatures, however, ablate tissue by denaturing all the molecules in the treated volume. This biothermal effect depends on the electrical parameters; it can elevate the tissue temperature to levels at which the cells become damaged, or it can result in only slight temperature increases that do not cause thermal damage to occur [Lavee, 2007]. The research group of B. Rubinsky found that within the family of electric fields that cause irreversible electroporation, there is a subset that minimizes Joule heating, resulting in temperature increases that stay below the threshold for thermal damage [Davalos, 2005]. To be succinct, this subset of electric fields is often referred to as "Non-Thermal Irreversible Electroporation" or NTIRE, designating electric fields that cause irreversible electroporation to occur without resulting in a level of elevated temperatures that can induce thermal damage.

Though this biophysical phenomenon is not yet completely understood [Teissie *et al*, 2005], electroporation is becoming extensively utilized in biotechnology and medicine. In the reversible mode, electroporation has become a central technology for cell manipulation [Neumann *et al*, 1982; Richter, *et al*, 1981], and, in combination with chemicals, it is considered promising for gene therapy [Titomirov *et al*, 1991; Heller and Heller, 2010], and is used clinically for electrochemotherapy [Snoj *et al*, 2007; Gargiulo *et al*, 2010]. It has been hypothesized that if certain electric pulses could be found that can irreversibly permeabilize the cell membrane without elevating the affected tissue temperature to levels that may induce thermal damage, then large volumes of tissue could be treated using non-thermal irreversible electroporation [Davalos *et al*, 2005]. This first mathematical study has proven that, while limited in range, such a domain of electric fields exists [Davalos *et al*, 2005]. Subsequent studies have shown that NTIRE can ablate tissue [Edd *et al*, 2006; Rubinsky *et al*, 2007; Rubinsky, 2007] while retaining the structural integrity of blood vessels, nerves, and extracellular matrix [Onik *et al*, 2007; Phillips *et al*, 2010] and that it is effective in destroying cancer in animal models [Al-Sakere *et al*, 2007; Ellis *et al*, 2011]. NTIRE involves the insertion of thin needle electrodes around an undesirable tissue or cell mass and the application of brief microsecond-scale electric pulses. The ability to apply NTIRE in a minimally invasive manner and the safety of this procedure [Thomson *et al*, 2011] have led to a recent surge in its clinical use.

1.2.2 A Historical Context for Irreversible Electroporation

Though the biomedical use of irreversible electroporation is a relatively new field, the area of electroporation has been under development since the mid 20th century, and the electroporation phenomenon has been noted (though most likely unrecognized at the time) in

scientific articles as early as the 18th century. A complete review of the development of irreversible electroporation over the years can be found in other sources such as [Ivorra and Rubinsky, 2010]. Here, a brief summary is given in order to place the development of irreversible electroporation in an historical context.

Irreversible electroporation may have received its first scientific observation as early as 1754 [Nollet, 1754]. In 1898, G.W. Fuller [Fuller, 1898] showed that high voltage discharges can kill bacteria in a water sample without inducing a significant increase in temperature [Ivorra and Rubinsky, 2010]. This and other examples indicate that the irreversible electroporation phenomenon was observed before the beginning of the 20th century. These studies, however, were merely observations and did not make the connection between cell death and an increase in cell permeability. Indeed, it was not until the second half of the 20th century that electroporation began to develop into a field. Sale and Hamilton are often cited as setting the basis of the field of irreversible electroporation. Through a series of publications examining the effect of high electric fields on microorganisms, they showed that the parameters resulting in cell ablation were the electric field magnitude and the time over which the electric field was applied [Sale and Hamilton, 1967]. This team showed that electroporation results when the transmembrane potential is increased to about 0.7 – 1.15 V, causing the cell membrane to change its structure and resulting in loss of the cell membrane semi-permeable state [Sale and Hamilton, 1968]. Through electron microscopy studies of e-coli and erythrocytes, Sale and Hamilton showed that cell death is caused by irreversibly affecting the cell membrane's ability to serve as a semi-permeable barrier [Hamilton and Sale, 1967].

The field of electroporation continued to develop throughout the end of the 20th century. Though the majority of this work was focused on reversible electroporation, these studies helped to develop and understand electroporation as a whole and aided future irreversible electroporation work. As noted by Ivorra and Rubinsky [Ivorra and Rubinsky, 2010] in their review, the first systematic study on the electrical parameters required for cell electroporation was perhaps done by U. Zimmerman's group, who measured the effect of pulse length and electric field amplitude on the cell membrane breakdown (in the form of how much intracellular content leaked out into the extracellular solution). They showed that at pulse lengths of 50-100 μ s, a critical electric field of 2.6 kV/cm is reached for human red blood cells and 2.8 kV/cm for bovine red blood cells at which maximal content leakage occurs, corresponding to a critical membrane potential of 1.1 V [Riemann *et al*, 1975]. In 1979, Pastushenko, Arakelyan, and Chizmadzhev published a series of studies concerning the electric breakdown of bilayer lipid membranes, developing the theory that permeabilization occurs due to the formation of transient pores [Abdior *et al*, 1979; Pastushenko *et al*, 1979a; Chizmadzhev *et al*, 1979; Pastushenko *et al*, 1979b; Arakelyan *et al*, 1979; Pastushenko *et al*, 1979c; Pastushenko *et al*, 1979d]. K. Kinosita and T. Tsong showed with osmotic mass transfer experiments on red blood cells the pore sizes can be varied in a controlled manner and that the membrane can reseal, incorporating foreign molecules into the intact erythrocytes [Kinosita and Tsong, 1977]. In the 1980s, the term "electroporation" was coined to describe this phenomenon when Neumann *et al*. [Neumann *et al*, 1982] used electrical pulses to transfect mouse lyoma cells with DNA. Additional applications for reversible electroporation began to develop in the form of cell fusion [Zimmerman, 1982] and introducing drugs such as bleomycin into cancer cells for cancer treatment (now referred to as electrochemotherapy) [Okino and Mohri, 1987]. During the 1990s, applications of reversible electroporation became further developed. Weaver and Langer's group developed a method for

transdermal drug delivery by electroporation [Prausnitz *et al*, 1993], and microbiology labs began to use electroporation as a standard method for gene transfections [Ivorra and Rubinsky, 2010]. L.M. Mir's group brought electrochemotherapy to clinical trials [Belehradek *et al*, 1993], and this method has now become an established method for treating cancer [Snoj *et al*, 2007; Gargiulo *et al*, 2010].

Though the field of electroporation had come a long way, the irreversible effects of electroporation were not utilized for medical applications until relatively recently. Though irreversible electroporation was utilized in the food industry to kill bacteria since the 1960s, from a biomedical viewpoint irreversible electroporation was, for the most part, regarded as an undesired effect. Most of the research into irreversible electroporation served to define it as an upper bound for reversible electroporation applications. In 2004, R. Davalos and B. Rubinsky proposed using IRE as a method to produce tissue ablation [Davalos and Rubinsky, 2004]. By choosing electrical parameters that minimized Joule heating effects, thermal damage to the tissue could be avoided and tissue ablation due solely to the cell membrane permeabilization could occur [Davalos, 2005]. It was at this point that the field of irreversible electroporation for medical applications really began to take off, and subsequent experiments on cells *in vitro* and animal models as well as clinical trials have advanced the field to its current state.

This introduction does not attempt to serve as a complete review of the numerous experimental and theoretical studies that have brought the field of electroporation to where it is today. More details may be found in many different reviews published in the field. Nonetheless, a quick review on practical applications of electroporation (Section 1.2.3) is given as well as a brief background on a theoretical model that have been developed to explain the electroporation phenomenon (Section 1.2.7), bringing some understanding to the mechanism by which electroporation acts. In addition, the development of irreversible electroporation with regard to cancer ablation and tissue engineering applications will be discussed in more detail in connection with the topic of this thesis (Section 1.3).

1.2.3 Applications for Electroporation

1.2.3.1 Applications for Reversible Electroporation

Reversible electroporation has been developed over the past 50 years for a variety of applications, ranging from gene transfer into cells in a lab setting to cancer treatment by electrochemotherapy. *In vitro* uses for reversible electroporation include loading genes by DNA transfection into cells [Gehl, 2003], cell fusion [Trontelj *et al*, 2008], and inserting proteins into the cell membrane by electroinsertion [Teissie, 1998]. Reversible electroporation has also been developed or investigated for *in vivo* uses such as transdermal drug delivery [Denet *et al*, 2004; Prausnitz *et al*, 1993], electrochemotherapy [Mir *et al*, 1998; Heller *et al*, 1999], and electrogene transfer for applications such as localized gene therapy [Mir *et al*, 1999] and delivering vaccinations by DNA transfer [Glasspool-Maolone *et al*, 2000]. More details on these and other uses of reversible electroporation can be found in review articles elsewhere.

1.2.3.2 Applications for Irreversible Electroporation

Irreversible electroporation has been developed for *in vitro* uses such as the sterilization of food and water as well as the extraction of intracellular components. Since 1961, irreversible electroporation has been used in the food industry for sterilizing and preprocessing food [Rubinsky, 2007]. More recently, Troszak and Rubinsky [Troszak and Rubinsky, 2011] investigated the feasibility of a singularity-induced micro-channel for the electroporation of water and other liquids with minimal power consumption. The formation of pores in the cell membrane has also been utilized for extracting intracellular components, and this too has been harnessed by the food industry as a means to extract juices from fruits and vegetables, sugar from sugar beets, oils from oil bearing plants, as well as additional products from microorganisms such as algae [Singh and Kumar, 2011].

This dissertation focuses on the *in vivo* application of irreversible electroporation for biomedical purposes. Irreversible electroporation is typically used *in vivo* for tissue ablation. Some areas that are currently being developed include:

1. Cancer treatment
2. Restenosis treatment
3. Tissue decellularization

The ablative modality of irreversible electroporation is being harnessed to target cancer tumors, resulting in cell death. In addition, the method of cell death by irreversible electroporation has been shown to result in a quicker recovery of the biological tissue, as discussed in the body of this thesis. Utilizing irreversible electroporation to treat restenosis has been investigated by Maor *et al.* [Maor *et al.*, 2008; Maor *et al.*, 2009]. Balloon angioplasty is a common procedure used to prevent blood vessel blockage, but, despite its wide use, approximately 40-60% of procedures result in increased arterial blockage later on in a process known as restenosis. Irreversible electroporation has been investigated as a technique used to ablate vascular smooth muscle cells in the artery, preventing them from proliferating and narrowing the artery. Finally, this thesis touches on an additional potential use of irreversible electroporation. Here, it is shown that when irreversible electroporation is applied *in vivo* to the artery, the tissue becomes naturally decellularized, leaving behind an intact extracellular matrix. This ability of irreversible electroporation to preserve the natural tissue scaffold could be harnessed for tissue engineering applications and developing natural tissue grafts. A more in depth background on cancer treatment and tissue decellularization are given in Section 1.3, as this is the main focus of this thesis work.

1.2.4 Mechanism of Electroporation

Though a comprehensive theory has not yet been developed to fully explain the mechanism of electroporation, extensive experimental work and proposed models have developed a strong foundation that has allowed for the development of electroporation for a wide array of applications and is currently being built upon to produce a more detailed understanding

of the phenomenon. The essential features of the electroporation process are known and can be briefly summed up as follows:

1. Electroporation utilizes short (on the order of μs to ms) electrical pulses, applying an elevated transmembrane potential. The cell membrane charges, and within a few microseconds, the transmembrane potential reaches a critical threshold (around $0.2 - 1 \text{ V}$).

2. The membrane conductivity increases immediately and a time dependent membrane transition occurs as long as the externally applied electric field is held at over above the critical value.

3. Depending on the electrical parameters utilized, once the external electric field is removed, either membrane stabilization and resealing occurs for reversible electroporation or loss of cell homeostasis leads to cell death by irreversible electroporation.

For reversible electroporation:

4. Once the electric field is lowered below the critical value, a stabilization process occurs over a few microseconds. The transmembrane potential drops quickly to near zero, and the membrane dramatically recovers to a level in which it is permeable to only small molecules.

5. The membrane reseals slowly over seconds or even minutes.

For irreversible electroporation:

4. Cell death occurs via a number of potential mechanisms such as continued pore growth and membrane rupture, membrane rupture due to colloidal-osmotic swelling, changes in ionic concentrations, and loss of cellular content. These mechanisms for cell death are described in more detail in Section 1.2.8.

The unique features of the lipid bilayer allow for this electroporation phenomenon to occur. The lipid bilayer consists of two layers of phospholipid molecules each with a hydrophilic triglycine head attached to two fatty acid hydrocarbon chains, as illustrated in Figure 1.1. In a cell, this lipid bilayer acts as a barrier between the interior of the cell and the extracellular medium, helping to regulate the passage of ions and molecules into and out of the cell. In contrast, the planar lipid bilayer is a simplified system developed in the laboratory setting that has often been used as a method for studying the effects of electroporation. When exposed to water, the phospholipids arrange themselves in the manner illustrated in Figure 1.1, forming a two-layered sheet. The polar heads on each side of the membrane are exposed to conducting solutions, and thus the membrane will act as a capacitor. The potential difference across a typical cell membrane is maintained at approximately 100mV , depending on cell type [Lewis, 2003]. When an external electric field is applied, however, ions in the extracellular and intracellular solutions will migrate toward the cell membrane. This produces an elevated transmembrane potential which can lead to the effects known as electroporation.

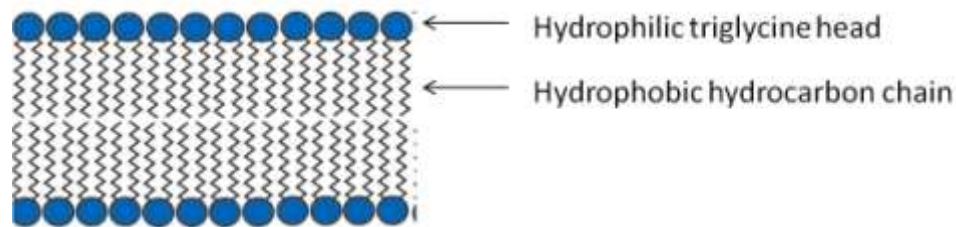


Figure 1.1. Schematic of a cell membrane lipid bilayer. The lipid bilayer, depicted here in cross section, consists of two layers of phospholipid molecules that arrange themselves such that the hydrophobic, hydrocarbon chains are oriented inward, protected from the aqueous surroundings, whereas the hydrophilic, triglycine heads form the border between the cell membrane and the surroundings.

It is widely accepted the electroporation causes hydrophilic pores to form in the cell membrane lipid bilayer. Though there has been little direct visualization of these pores due to the chemical composition, thickness, and fluid nature of the bilayer membranes [Waver and Chizmadzhev, 1996], indirect methods support the presence of the hydrophilic pores. The theory and mechanism of this pore formation process is described in more detail in Section 1.2.7. Briefly, the main steps for pore formation are as follows:

1. Short electrical pulses charge the membrane lipid bilayer as a result of ion flow.
2. Thermal fluctuations in the lipid bilayer cause briefly-lived hydrophobic pores to form.
3. Hydrophobic pores transition into stable, water-filled hydrophilic pores, resulting in a tremendous increase in ionic and molecular transport into the cell.
4. If the electrical parameters cause reversible electroporation, the hydrophilic pores close rapidly and then slowly seal over time. If irreversible electroporation occurs, the pores may never completely seal, and the cell is ablated.

This aqueous pore theory explains many of the experimental observations pertaining to electroporation that have been documented over the past 50 years. Experimental work builds the backbone of electroporation knowledge and has helped to develop this field to its current level, enabling electroporation to be commonly utilized in the lab as well as harnessed in the medical field for applications such as cancer treatment. From this large set of work, the field has gained knowledge about what effect the electrical parameters have on the electroporation process and how pores form and develop. The next sections give a brief summary of some of the important fundamental knowledge gained from this type of work, helping to understand the effect of the different electroporation parameters as well as the dynamics of pore formation.

1.2.5 Electrical Parameters

1.2.5.1 Electric Field

The applied external electric field is very important in electroporation because it causes an initial transmembrane potential to form and thus affects pore formation. Thus, it is critical that the applied electric field is high enough that the transmembrane potential can surpass a critical threshold of 0.2- 1 V (depending on cell type and experimental conditions) for the process of electroporation to occur. The electric field has been shown to affect 1) pore size, 2) fraction of long-lived pores, and 3) location of pore formation.

By studying the ability to introduce different sized molecules into human erythrocytes, Kinoshita and Tsong showed that an increase in the applied electric field from 2.2 kV/cm to 3.7 kV/cm could be used to obtain larger pores in the cell membrane [Kinoshita and Tsong, 1977]. This was also demonstrated by Huang and Rubinsky by measuring the current for a single cell captured in a micro-chip, showing an increase in current with pulse amplitude, indicating a potential increase in pore size with applied electric field. [Huang and Rubinsky, 1999].

In addition, an increase in the electric field results in the development of a greater fraction of long-lived pores. This is because the critical voltage is reached for a larger area of the cell membrane due to a higher energy available for pore formation. This was shown by Miklavcic's group by combining a simple theoretical model with experimental results [Pavlin *et al.*, 2007]. The electric field is also expected to affect the total area of the membrane that undergoes electroporation [Rosemberg and Korenstein, 1990].

The electric field is also important in that it influence the location of pore formation. Pore formation in the cell occurs in an asymmetric fashion. When an external electric field is added to the resting potential of the cell membrane, the side of the cell facing the cathode becomes depolarized whereas the side of the cell facing the anode results in a state of being hyperpolarized [Ben-Or and Rubinsky, 2010]. Tekle *et al.* [Tekle *et al.*, 1994] studied this asymmetric pore formation by observing the transport of Ca^{2+} and three different DNA indicators by means of electroporation through different cell types. From their molecular transport data, they were able to show that both sides of the cell were permeable in different manners. They concluded that the anode side of the cell developed a greater number of small pores in the cell membrane, whereas the cathode had a smaller population of larger pores. Another group used fluorescent dye to image Chinese hamster ovary cells during electroporation [Gabriel and Teissie, 1997]. Pore formation was shown to occur only on the anode side when a lower electric field was applied. However, when the electric field was increased to a higher threshold, both the cathode and anode side became permeabilized, supporting the observations of Tekle *et al.* and others that the electric field affects the location of pore formation and that pores develop in the cell membrane in an asymmetric fashion. This asymmetric pore formation phenomenon and dependence on electric field magnitude was also shown for sea urchin eggs by measuring the change in membrane conductance during the course of electroporation [Kinoshita *et al.*, 1988].

Tekle *et al.* [Tekle *et al.*, 1994] theorized that this phenomenon of asymmetric pore formation occurred due to the vector sum of the resting potential and the membrane potential induced by the applied electric field. The transmembrane potential reaches the threshold value

first on the anode side of the cell, and the pores form slowly since the transmembrane potential is only slightly above the critical value for pore development. Thus, as more pores form, the anode side of the cell becomes more conductive, resulting in an increased potential difference across the cell membrane on the cathode side of the cell. This higher transmembrane potential causes rapid pore expansion on the cathode side. However, since the pores form rapidly the transmembrane potential will also quickly drop, preventing additional pores to form and resulting in the observed asymmetric effect.

1.2.5.2 Pulse Length

While the electric field was shown to influence the location of pore formation, the extent of pore formation is dependent on the length of the applied pulse. Kinosita and Tsong showed that an increase in the pulse duration resulted in the formation of larger pores in the cell membrane of human erythrocytes [Kinosita and Tsong, 1977]. By maintaining either a constant electric field and increasing pulse duration or increasing the electric field for constant pulse lengths, they showed that the pore size would increase with either electric field or pulse length. Rosemberg and Korenstien also supported this with their work on giant photosynthetic membrane vesicles, using a voltage-sensitive optical probe to conclude that an increase in the electric field pulse duration resulted in an increased area for a single pore [Rosemberg and Korenstein, 1990].

1.2.5.3 Number of Pulses

The number of pulses increases the number of stable pores that form in the cell membrane. This was shown by Miklavcic's group, suggesting that each additional pulse allows more or larger pores to form without affecting the number of transient or short-lived pores that are present in the cell membrane [Pavlin *et al.*, 2007]. Increasing the number of pulses has also been thought to correlate with an increase in the pore lifetime, resulting in molecular transport through the cell membrane over the course of the electrical pulse protocol, as supported by Gehl and Mir's work in examining the effect of different electroporation parameters on gene transfection [Gehl and Mir, 1999].

1.2.5.4 Pulse Frequency

Considerations of pulse frequency are important for avoiding thermal damage to the cell. By utilizing an interval between pulses, the cell or biological tissue being electroporated can have a chance to cool down between pulses, keeping the tissue temperature at a minimum. Thermal damage considerations in relation to Joule heating are discussed in more detail in Section 1.2.9.

1.2.5.5 Temperature

The temperature that the cell is held at during electroporation can also play an important role in how electroporation occurs. Though most experimental results are obtained at physiological temperatures, it is necessary to keep in mind that operating under different temperatures can severely affect the outcome. For example, Kinosita and Tsong's experiment with human erythrocytes showed that at 37 °C, the cells quickly regained their impermeability to cations after the external electric field was removed, indicating that the pores were able to close quickly. However, when the temperature was brought down to 3 °C the cells were still

permeable at 20 hours after electric field was removed [Kinosita and Tsong, 1977]. The temperature has a strong affect on membrane fluidity, and lowering the physiological temperature results in a cell membrane that is less fluid causing a decrease in permeabilization [Kanduser et al, 2008] as well as an increase in pore sealing time. Knowledge of temperature dependence is not only important in predicting membrane behavior during electroporation, but some researchers have looked into harnessing this effect for focused tissue ablation, combining cryosurgery and electroporation to target a specified area of tissue [Daniels and Rubinsky, 2011].

1.2.6 Transmembrane Potential and Pore Dynamics

1.2.6.1 Transmembrane Potential

The applied electric field affects the transmembrane potential that builds up across the cell membrane, resulting in the formation of electropores. The transmembrane potential has been measured using voltage-sensitive dye on the cell membrane combined with digital video microscopy [Ho and Mittal, 1996]. When an external field is applied, positive and negative charges within the cell accumulate at locations on the cell membrane closest to the electrode cathode and anode. Thus, the potential across the cell membrane is location dependent and varies along the cell. The lipid bilayer is approximately 5 nm thick and, combined with the membrane's capacitance-like properties, this amplifies the external electric field. For an applied electric field across a cell, the transmembrane potential has been modeled as:

$$U = f_c g r E \cos(\theta) [1 - e^{-t/\tau}] \quad (1.1)$$

where f_c is the cell shape factor, g is the relative electric permeability of the membrane, E is the applied electric field in units of V/m, r is the cell radius (in meters), θ is the angle between the electric field and the point of interest on the cell membrane, t is the time after the electric field was first applied (in seconds), and τ is the time constant of the cell membrane [Ho and Mittal, 1996]. Here, g is a function of the electrical conductivities of the extracellular and intracellular mediums as well as the cell membrane.

The membrane charging time is approximately 1 μ s, and for typical electroporation procedures, this is much smaller than the length of the applied electric pulse (typically 70-100 μ s for many irreversible electroporation applications). In this case with ($\tau \ll t$), the cell membrane can be considered as a pure dielectric with a relative electric permeability of $g = 1$. Thus, the transmembrane equation can be simplified. Since the electric pulse length is much longer than the membrane charging time, the exponential term drops out. For a spherical cell with a shape factor of 1.5, the resulting transmembrane potential can be calculated as:

$$U = 1.5 r E \cos(\theta) \quad (1.2)$$

For an elongated cell with a shape factor of $f = 0.5$, the maximum transmembrane potential for a cell oriented parallel to the electric field can be estimated as:

$$U_{\max} \approx 0.5 E L \quad (1.3)$$

where L is the characteristic length of the cell in the longer direction [Ho and Mittal, 1996].

Going back to the spherical cell transmembrane potential (Equation 1.2), the amount that the external electric field (E) is amplified at the poles ($\theta = 0, \pi$) to result in the transmembrane electric field (E_m) can be approximated as:

$$E_m = 1.5rE/h \quad (1.4)$$

where h is the cell membrane thickness (approximately 5 nm for a typical cell) [Weaver and Chizmadzhev, 1996]. For a spherical cell of 10 μm in diameter, the transmembrane electric field is approximately 1,500 times that of the external electric field.

Though Equation 1.2 can be very useful in estimating the transmembrane potential corresponding to a given applied electric field and has been shown to correlate well with experimental data, experiments have also demonstrated that this equation is no longer valid once a significant pore population has developed [Weaver and Chizmadzhev, 1996].

The transmembrane potential needed to induce electroporation has been determined experimentally for a variety of membranes and cell types. In studying the kinetics of membrane permeabilization on isolated rat skeletal muscle cells, Bier *et al.* determined that when an electric field was applied, transient pores formed when the transmembrane potential reached approximately 300-350 mV [Beir *et al.*, 1999]. A range of critical transmembrane potentials have been obtained by others, depending on cell type. The threshold value for electroporation has been estimated to be approximately 0.2 – 0.25 V [Teisse and Rols, 1993] and up to 1 V [Sale and Hamilton, 1968].

1.2.6.2 Pore Dynamics

Much of the knowledge base on electroporation has come from experimental studies on planar lipid bilayers and cells. From these observations on how pores form and progress, models have been developed to explain and predict the electroporation process. Understanding the pore dynamics is important in developing more in-depth theories that can be used as a method to design electroporation parameters to achieve a desired effect, be it introducing certain drugs into the cells or ablating the cells for cancer treatment. Here, some experimental results about pore development are given. The aqueous pore theory described in Section 1.2.7 is able to explain many of these observed phenomena.

As discussed earlier, it has been theorized that since the transmembrane potential is highest at those areas closest to the electrodes, the maximum number of pores will be created in those areas of the cell membrane. Chang and Reese used rapid-freezing electron microscopy to examine human red blood cells during electroporation, showing volcano-shaped pores that expanded rapidly to 20 – 120 nm within the first 20 ms [Chang and Reese, 1990]. In order to obtain these results, they used an electrical protocol of a 4-5 kV/cm electric field, a pulse length of 0.3 ms, and at a frequency of 100 kHz. They noticed that the variation in pore size changed with time during and after the electroporation procedure, and hypothesized that the process of electroporation consists of three stages [Ho and Mittal, 1996]:

1. Pore formation approximately 3 ms after applying the electroporation pulse.
2. Pore expansion at 20 ms after pulse application.

3. Pore shrinkage and resealing over several seconds after the electrical pulse has been removed.

Kinosita *et al.* [Kinosita *et al.*, 1992] showed similar stages of electroporation using fluorescent dye on the sea urchin egg's cell membrane. They, however, had events occurring at a different time scale than that observed by Chang, observing pore formation within 2 μ s. With a continued electric field, an increase in the size and number of pores occurred in the order of microseconds. Similar to that obtained by Chang, Kinosita *et al.* observed that the cell membrane took several seconds in order to recover after the electrical pulse had been removed.

The dynamics of pore sealing were investigated in more detail by Saulis *et al.* [Saulis *et al.*, 1991]. They divided the pore resealing process into three distinct stages:

1. The removal of the external electric field resulted in a rapid drop of pore size in less than 1 sec.
2. The pore size decreased slowly over several minutes. It is hypothesized that this is due to the presence of an additional energy barrier that must be overcome in order for small pores to close.
3. Complete closing took over 10 minutes to occur. This is believed to be due to energy barriers present in converting from a hydrophilic pore to a hydrophobic pore.

Chernomordik and colleagues [Chernomordik *et al.*, 1987] measured the conductance through human erythrocytes and L-cell membranes to show two stages of pore resealing. During the first stage, the conductance decreased rapidly (< 1 ms) due to a decrease in the transmembrane potential. During the second stage of pore resealing, the conductance decreased on a slower timescale (seconds to minutes), corresponding to a decrease in the number and radius of the pores and resulting in complete sealing of the cell membrane. Pore formation and membrane resealing depend on a variety of parameters such as the electrical protocol and temperature [Kinosita and Tsong, 1977] and is affected by additional factors such as the cytoskeleton [Teissie and Rols, 1994].

In addition to the timescale of pore formation and sealing, studies have examined the pore population and amount of area that becomes permeable due to electroporation. Rosemberg and Korenstein [Rosemberg and Korenstein, 1990], used electrophotoluminescence to quantify pore size and area, showing the formation of reversible pores of less than 5.8 nm in radius occupied only 0.075% of the total membrane surface area.

The aqueous pore model is the most accepted model for predicting pore size and formation, offering an explanation for the mechanisms of electroporation. This model is able to predict many of these observations that have been made experimentally, and a brief overview of this pore formation model is given in the following section.

1.2.7 Pore Formation: The Aqueous Pore Theory

Electroporation is often also referred to as electropermeabilization. This is because the exact method by which the cell membrane reorganizes and results in an increased membrane conductance and molecular flow into the cell is still unknown. Nonetheless, a great deal of experimental data supports the hypothesis that pores form in the cell membrane, supporting the label “electroporation”. Here, the most commonly accepted theory of pore formation due to electroporation is presented. This pore energy theory (often referred to as the aqueous pore model) was originally developed to explain experimental observations for pores in lipid bilayer membranes and predict how pore-formation and expansion occurs. Though this theory was originally developed for a planar lipid bilayer membrane, it can help to give an understanding of how pore formation may occur in the lipid bilayer of the cell membrane. In addition, others have expanded on this theory to give a description of pore dynamics tailored for the cell.

Briefly, when an electric field is applied across the cell membrane, large transmembrane potentials cause thermal fluctuations in the lipid bilayer to occur. These fluctuations create initial, hydrophobic pores in the membrane, as illustrated in Figure 1.2a. These pores are very unstable, and in this configuration, they will only last as long as a few lipid fluctuations before disappearing. Depending on the magnitude and duration of the external electric field, however, these pores may expand. If the radius of the pores passes a critical radius threshold (about 0.3 – 0.5 nm), the pores will rearrange themselves to form hydrophilic pores (Fig. 1.2c). These hydrophilic pores are much more stable, and it is during this phase that drugs and macromolecules can be introduced into the cell for reversible electroporation purposes. Once the electric field is removed, these pores may shrink and eventually reseal. For irreversible electroporation, however, stronger electrical parameters may result in cell death through a number of mechanisms such as pore expansion or loss of cell homeostasis. These mechanisms of cell death by irreversible electroporation are described in more detail in Section 1.2.8.

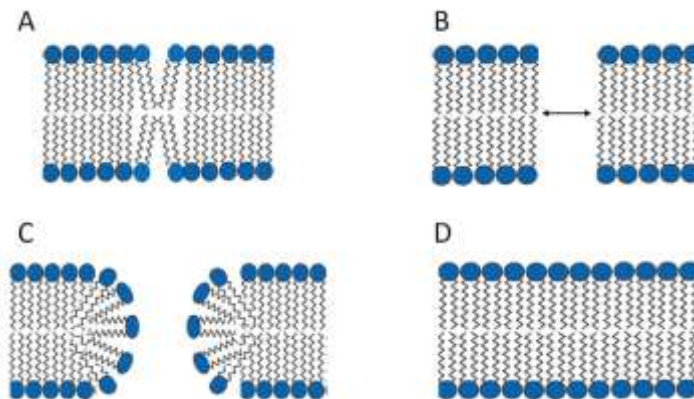


Figure 1.2. Pore creation by electroporation. a.) Thermal fluctuations occur in the lipid bilayer membrane. b.) If a high enough electric field is present, a hydrophobic pore is created that is able to expand. c.) Once the pore hydrophobic pore reaches a critical radius, the lipids will rearrange themselves to form a more stable hydrophilic configuration. d.) For reversible electroporation, the pore will shrink and eventually reseal after the external electric field has been removed.

The theory of pore formation may be used to explain questions such as why pores are able to form stable hydrophilic pores with the addition of an external electric field and the trend of pore formation and resealing. As described by Weaver and Chizmadzhev as well as Glasar [Weaver and Chizmadzhev, 1996; Glasar *et al*, 1988], the free energy due to the spontaneous formation of a cylindrical hydrophilic pore is developed based on a gain of edge energy for the pore and reduced due to the loss of a cylindrical section of the membrane where the pore forms. This pore formation energy can be given as:

$$\Delta W_p = 2\pi\Upsilon r_p - \pi\Gamma r_p^2 \quad (1.5)$$

where r_p is the radius of the pore, Υ is the edge energy at the pore walls, and Γ is effective tension of the membrane. The first term on the right represents a gain in edge energy due to the formation of a pore of radius r_p , whereas the second term includes a reduction of energy due to the loss of membrane in the circular region where the pore develops. This results in a parabolic energy barrier that must be over-come for hydrophilic pore formation where the maximum occurs at the critical radius for hydrophilic pore creation as illustrated in Figure 1.3. This energy barrier is high enough such that it is very improbable that random lipid fluctuations will cause spontaneous hydrophilic pore formation. Adding an externally applied transmembrane potential across the membrane, however, causes the energy barrier to decrease. The hydrophilic pore can be treated electrically as having a change in energy as the lipid is replaced by water to form the pore due to the change in specific capacitance, C_{LW} .

$$C_{LW} = \left(\frac{\epsilon_w}{\epsilon_m} - 1 \right) C_o \quad (1.6)$$

Here ϵ_w is the permittivity of pure water, ϵ_m is the permittivity of the lipid interior of the membrane, and C_o , the capacitance per unit area of a pore-free membrane of thickness h , is equal to ϵ_m/h . Including this extra term into the energy barrier equation (1.5) gives the free energy for pore formation as a function of the radius of the pore and the spatially averaged transmembrane voltage, U :

$$\Delta W_p(r_p, U) = 2\pi\Upsilon r_p - \pi\Gamma r_p^2 - 0.5C_{LW}U^2\pi r_p^2 \quad (1.7)$$

As can be seen here and in Figure 1.3, increasing the transmembrane voltage lowers both the energy barrier and the critical radius for hydrophilic pore formation. Thus, the higher the electric field and resulting transmembrane voltage, the higher the probability of pore formation and growth.

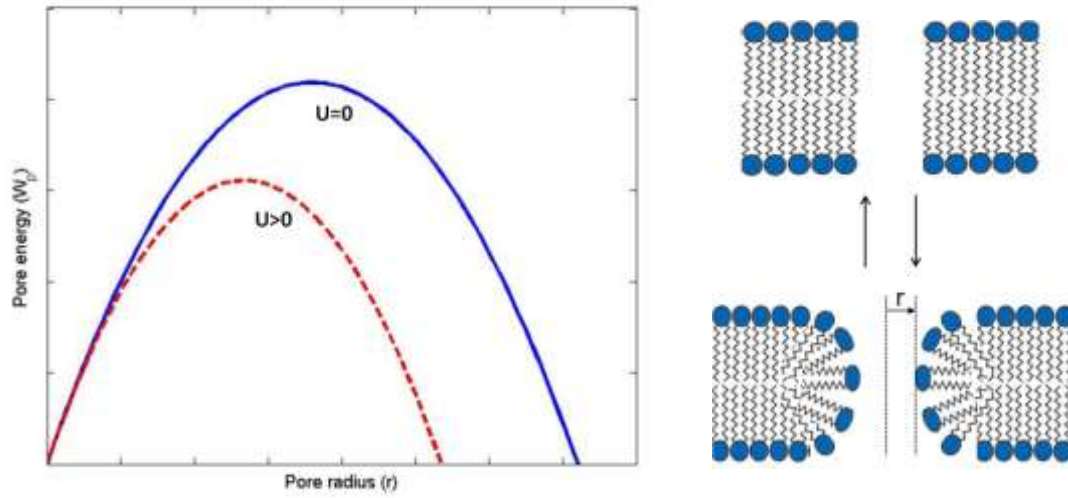


Figure 1.3 Model for energy of hydrophilic pore formation illustrates the relationship between pore energy and pore radius. An increase in the transmembrane potential results in a decrease in the energy barrier for hydrophilic pore formation. Here, this is illustrated for the case when there is no applied transmembrane potential ($U = 0$) and for an elevated transmembrane potential case ($U > 0$).

The critical pore radius, corresponding to the maximum barrier energy can be given as:

$$r_c = \frac{\gamma}{\Gamma + 0.5C_L W U^2} \quad (1.8)$$

This model gives a strong basis for understanding how applying an external field can lead to pore formation in the liquid bilayer. However, it must be noted that this simplified model neglects to take some important effects into account. First, it is an oversimplification that the edge energy (γ) will remain constant. Rather, it is expected that the edge energy will increase as the pore radius decreases [Weaver and Chizmadzhev, 1996]. In addition, in order to account for the hydrophobic nature of pores before they transition to stable hydrophilic pores, another energy branch corresponding to hydrophobic pores should be added to the model. Including these two factors adds an additional degree of complexity to the model. Thus, this model now utilizes a first energy branch (W_1) that is a modified version of that given in Equation 1.7 for the hydrophilic pore as well as a second energy branch (W_2) to account for the presence of hydrophobic pores prior to transition [Weaver and Chizmadzhev, 1996]. Figure 1.4 illustrates this pore energy – radius relationship.

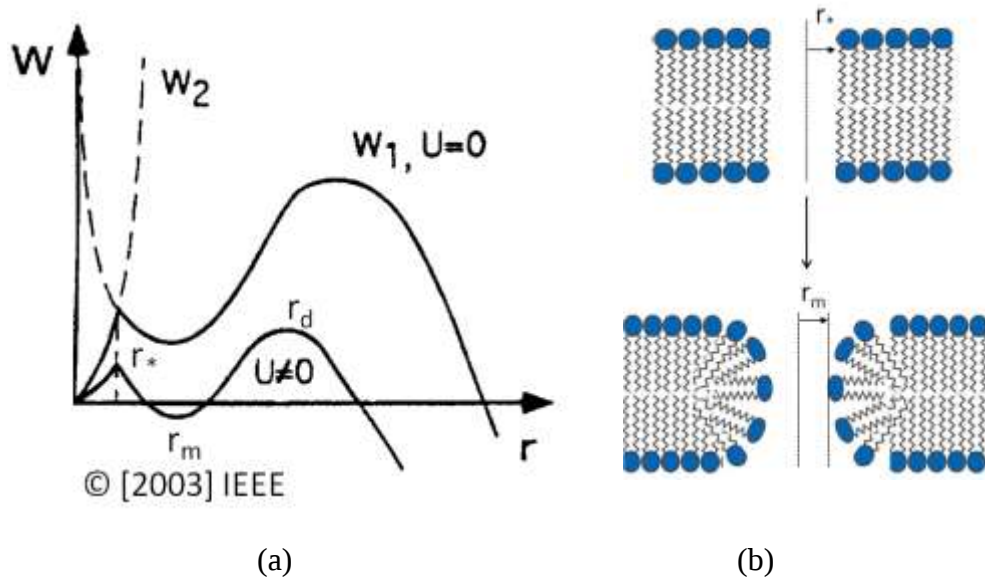


Figure 1.4. Relationship between pore energy and pore growth. (a) The energy of a hydrophobic pore is shown to the left of r^* , whereas the pore energy branch for the growth of a hydrophilic pore is shown to the right. As illustrated in the plot (a) and in the schematic (b), when a hydrophobic pore reaches a radius of r^* , it has reached the energy barrier for hydrophilic pore creation. A stable hydrophilic pore then forms with a radius of r_m . If additional energy is added to the system, the pore will continue to grow. Should the pore exceed a critical radius of r_d , the pore will expand spontaneously with no additional energy input needed. Plot (a) is reprinted with modifications from [Weaver, 2003].

A quick look at the model illustrated in Figure 1.4 offers an explanation for some of the key features observed during the electroporation phenomenon and postulated by the aqueous pore theory. As can be seen, hydrophobic pores (defined by the energy branch to the left of r^*) are at all times unstable. These result from thermal fluctuations in the cell membrane, and if no additional energy is added to the system, the pore closes quickly. Once enough energy has been added to the system for the pore to reach a radius of r^* , however, a new energy minimum occurs allowing for the formation of stable hydrophilic pores. Additional energy is needed from here to maintain pore growth. Pores to the left of r_d are still reversible, and if the external energy is removed, their radius will decrease quickly down to r_m . However, should the radius exceed the a critical threshold of r_d , the pore will begin to expand spontaneously, explaining the threshold phenomenon observed experimentally in which a jump in membrane conductance occurs followed by membrane rupture [Chernomordik *et al*, 1983; K Neu and Neu, 2010].

The transient aqueous pore model described here is able to successfully predict some of the key features of electroporation. As described by Weaver [Weaver, 1995] and Chen *et al*. [Chen *et al.*, 2006], the following behavior characterizes electroporation:

1. Membrane rupture is stochastic in nature with a probability of rupture associated with the transmembrane potential [Chen *et al.*, 2006]

2. A critical transmembrane voltage (U_c) increases the probability of rupture occurring [Weaver and Mintzer, 1981].
3. A given fraction of the cell membrane area becomes permeable [Rosemberg and Korenstein, 1990].
4. The occurrence of either irreversible electroporation or reversible electroporation is dependent upon the amplitude of the applied electric field [Benz *et al.*, 1979].

The aqueous pore model is able to explain these key features. For example, the stochastic nature of rupture is explained in the model by the diffusive escape of very larger pores [Abidor *et al.* 1979]. In addition, the average value of the critical transmembrane potential is reasonably predicted [Weaver, 1995]. The model predicts that less than 0.1 % of the cell membrane develops pores [Freeman *et al.*, 1994], and this was shown to be the case experimentally [Rosemberg and Korenstein, 1990]. The model also has been shown to predict the transition between membrane destruction and reversible electrical breakdown is dependent on the membrane properties as well as the electric field amplitude and pulse duration [Barnett and Weaver, 1991].

The aqueous pore model as described here has been developed for a lipid bilayer membrane. Some groups have developed more complicated analytical models to explain, for example, electroporation for a spherical cell [Krassowska and Filev; Joshi *et al.*, 2004]. Krassowska and Filev's model illustrates a negative feedback between pore creation and the transmembrane potential, predicting that pores will not be able to expand spontaneously (as predicted from the lipid bilayer membrane model) and that IRE must occur by some other mechanism [K Neu and Neu, 2010]. Additional details on the aqueous pore model and other variations and revised effects of this model that have been incorporated into it by various researchers can be found elsewhere. In conclusion, the aqueous pore model is widely accepted for describing transient pore developed during electroporation. Nonetheless, more work is warranted in order to develop this model for describing electroporation of cells in tissues.

1.2.8 Mechanisms of Cell Death by Irreversible Electroporation

From the aqueous pore model described in Section 1.2.7 we know that hydrophobic pores occur due to thermal fluctuations in the lipid bilayer, and that these pores rearrange to a hydrophilic configuration once a critical pore radius is reached. Earlier sections also looked at experimental results and predictions on the pore dynamics. Most of these studies are based on developing an understanding of reversible electroporation. These are useful in adding to the field of electroporation as a whole and can help in understanding how pores form and develop for situations of irreversible electroporation as well. From a cell ablation point-of-view, however, perhaps one of the most important areas to understand is the mechanisms by which cell death by irreversible electroporation occurs. Krassowska and Neu [K Neu and Neu, 2010] categorizes these mechanisms into four different methods as follows:

1. Membrane rupture due to the creation of a supercritical pore that expands spontaneously

2. Membrane rupture resulting from colloidal-osmotic swelling
3. Cell death due to irreversible changes in ionic concentrations in a large number of long, living, small pores
4. Loss of cellular content due to the presence of one or several giant pores that develop as a result of post-shock coarsening

As can be seen, an understanding of the number and sizes of pores that develop is essential for determining how irreversible electroporation occurs. Also, as pointed out by Krassowska and Neu, the occurrence of each mechanism depends of competing factors.

For the first method, membrane rupture due to a spontaneously expanding pore is aided by cell deformation due to the applied electric field. The electric field causes electric stresses to form at the cell membrane interface, causing the cell to deform. The membrane will stretch until equilibrium is reached between the electric stress and the increased surface tension of the cell membrane [Isambert, 1998]. This higher surface tension, in turn, adds energy to the pore expansion process. On the other hand, increased pore size has been shown to actually lower the surface tension, which may partial or completely counteract the tension increase from cell deformation [Isambert, 1998]. In addition, the mechanism of membrane rupture by spontaneous cell deformation may be stopped due to a negative feedback mechanism. An increase in pore size will cause the conductance across the cell membrane to increase. The increased conductance will drive the transmembrane potential down. A lowered transmembrane potential will slow down or even reverse the growth of the pores, keeping the pores from expanding to the point of rupturing the cell membrane. Thus, for spontaneous pore expansion and membrane rupture to result in cell death, it needs to occur during the early stages of electroporation before this negative feedback mechanism can kick in [K Neu and Neu, 2010].

For the second method, a large number of small pores that are permeable to water and ions but not macromolecules may result in membrane rupture by colloidal-osmotic swelling. Colloid-osmotic lysis occurs when the equilibrium of ions through small pores around the size of 0.5-1.0 nm radius causes additional water to also enter the cells with the ions [Knowles and Ellar, 1987]. This results in cell swelling, leading to cell death. These small pores that induce colloidal-osmotic swelling are too small to allow macromolecules such as nucleic acids and proteins out of the cell, increasing the internal osmotic pressure [Knowles and Ellar, 1987]. This method of cell death was observed by Kinosita and Tsong [Kinosita and Tsong, 1977] when they applied a single 3.7 kV/cm electric pulse with a 20 μ s pulse length to human erythrocytes. The cells continued to swell after electroporation until the cell volume became 155% of the original volume, tearing the cell membrane [Tsong, 1989]. The pores that fit within this radius range, however, seal with the same time scale as seen for cell swelling, and thus pore sealing may counteract this mechanism of cell death [K Neu and Neu, 2010].

When a large number of small pores are able to stay open, changes in the ionic concentrations within the cell can occur as well as loss of intracellular content, and this in and of itself may lead to cell death by changing the content of the cell to a level from which it cannot recover. Ion transport may also be increased by using longer pulses or adding additional pulses to the electroporation protocol. As with the second method described above, however, these

small pores may reseal, potentially preventing this mode of cell death from occurring [K Neu and Neu, 2010].

Finally, the last mechanism of cell death by electroporation described here involves the occurrence of several very large pores that develop due to post-shock coarsening. This refers to the case when one or a few pores expand to a radius much larger than the rest of the pores. These giant pores have been visualized by others. Tekle et al. [Tekle *et al*, 2001] used fluorescent dye to visualize phospholipid vesicles with a standard fluorescent microscope and showed that a single pore of about 7 μ m developed due to electroporation on the cathode side, whereas the anode side developed many small pores. Zhelev and Needham [Zhelev and Needham, 1992] were able to produce stable pores of up to one micrometer in giant liposomes. They showed the presence of one giant pore with a lifetime of up to several seconds. These large pores allow for the loss of cellular content due to membrane tension or osmotic pressure, leading to cell death. Loss of cell content, however, causes the cell size to decrease. This acts as a competing factor, encouraging the large pores to shrink and reseal. Colloidal-osmotic swelling, however, may help to counteract giant pore leak out, preventing the cell from shrinking and aiding in cell death [K Neu and Neu, 2010].

As can be seen, these different mechanisms can occur with varying degrees, contributing to cell ablation. The dominating mechanism may be dependent on cell type, surrounding conditions, and experimental parameters. Due to the complexity of this issue, the many different factors that go into determining the final outcome, and the dependence on cell type and experimental conditions, it has been difficult to develop models that predict irreversible electroporation for medical treatment applications. Current models may be useful for understanding how electroporation occurs, but they do not take into account many effects, even at the single-cell level. When using irreversible electroporation for tissue ablation, an entire additional level of complexity is added; now, cells interact with each other and the extracellular matrix, and a full understanding of these interactions is still being sought out. Further development of these in-depth theoretical models is needed. In the meantime, pre-treatment modeling utilizes predetermined experimental results for the electric field threshold shown to cause irreversible electroporation for the specific tissue of interest. Finite element models are then utilized to examine the electric field distribution resulting from factors such as the electrode geometry, applied electroporation parameters, and the tissue electrical properties. Based on experimental results, an acceptable electric field range is desired for cell ablation by irreversible electroporation. These finite element models are used to optimize electric parameters and electrode setups for the treatment scenario in order to cause cell ablation in a specified volume of tissue.

1.2.9 Joule Heating to Biological Tissue and Non-Thermal Irreversible Electroporation

Irreversible electroporation utilizes electrical pulses to ablate cells. It is well known that high voltage electrical shock can cause extensive damage to tissue, injuring nerves, skeletal muscle, and blood vessels through Joule heating, electroporation mechanisms, or both [Tropea and Lee, 1992]. A pure electroporation injury affects on the cell membrane, resulting in cell death often due to loss of cell homeostasis and osmotic swelling. Thermal burn injuries, however, lead to both cell membrane disruption and protein denaturation due to Joule heating

[Lee and Despa, 2005]. It is important, when applying irreversible electroporation to tissue for cell ablation, to be able to separate these effects. The amount of Joule heating that occurs during irreversible electroporation depends on both the applied electric field and the pulse duration. Biological cells and the extracellular matrix are sensitive to temperature, and, in order to promote tissue recovery and continued function after electroporation, it is often advantageous to develop an electrical protocol that utilizes electric pulses for cell membrane permeabilization while avoiding thermal damage to the tissue due to Joule heating. This concept is especially important in the context of this thesis work, where the ability of critical and delicate tissues to regain structure and function after electroporation is investigated. In addition, the potential development of a decellularized tissue scaffold is examined, and minimizing Joule heating keeps the extracellular matrix from being thermally damaged by the electroporation protocol. Here, a brief overview of tissue thermal damage in regard to irreversible electroporation is given. In the context of this work, non-thermal irreversible electroporation (NTIRE) is used to describe an electroporation protocol that results in minimal Joule heating to the tissue and avoids thermal damage.

Thermal damage to biological tissue is both temperature and time dependent. This dependency is illustrated as an example in Figure 1.5.

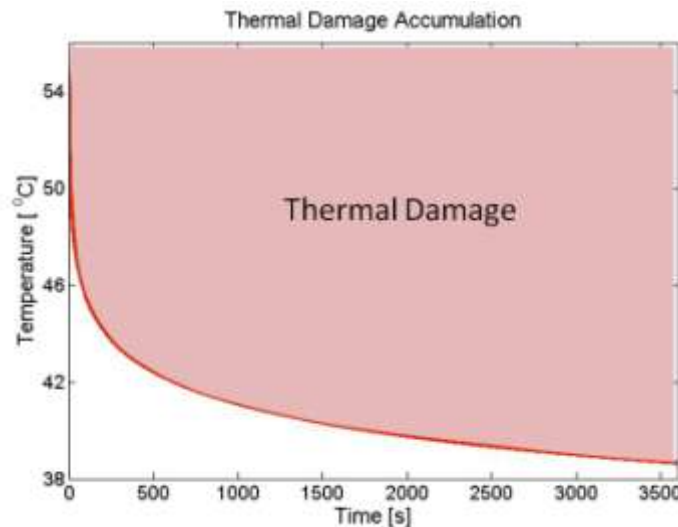


Figure 1.5. An example of how the probability of thermal damage to the tissue depends on both time and temperature. Here, the probability of 5% of the tissue becoming damage is plotted as a function of time and temperature for arterial tissue. As can be seen, over long periods of time, even a small increase in temperature can cause damage to occur.

The molecular dynamics associated with thermal damage to the cell has reaction dynamics resembling a first-order chemical reaction, indicating that Maxwell-Boltzmann statistics can be used to describe the rate at which biological tissue is converted from viable to thermally damaged [Lee, 1991]. This single barrier model is illustrated in Figure 1.6.

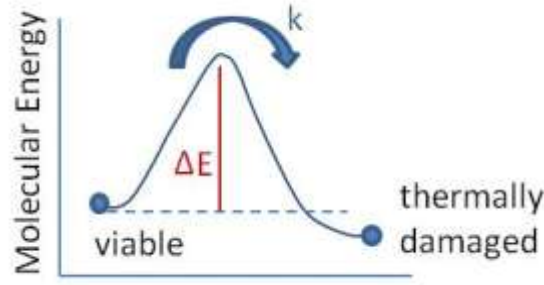


Figure 1.6. Illustration describing the single barrier model for the process of how tissue will go from viable to a state of being thermally damaged. This process is not spontaneously reversible, and the rate of conversion to a thermally damaged state is given by the Arrhenius equation [Tropea and Lee, 1992].

The rate at which tissue becomes thermally damaged is described by the Arrhenius equation:

$$K = A \exp\left(\frac{-\Delta E}{RT}\right) = \frac{d\Omega}{dt} \quad (1.9)$$

where ΔE (the energy barrier height) and A (the rate of cell membrane damage accumulation) are tissue dependent parameters, R is the ideal gas constant, and Ω is used to describe the probability of membrane damage. Thus, the thermal damage over a given pulse length can be quantified as:

$$\Omega = \int_0^t A \exp\left(\frac{-\Delta E}{RT(t)}\right) dt \quad (1.10)$$

After analyzing the temperature distribution in the tissue that would occur due to Joule heating effects of electroporation, an estimate of the probability of thermal damage can be obtained. Since the temperature distribution will be changing with time, this thermal damage integral provides a measure of the damage accumulation. By modeling the electrical parameters and tissue geometry in advance, electrical parameters can be chosen that minimize thermal damage allowing for cell ablation solely due to cell membrane permeabilization effects. Figure 1.7 illustrates how the local electric field and pulse duration can determine the type of effect electroporation has on the cell. In order to obtain NTIRE results, the parameters must be strong enough to cause irreversible electroporation to occur yet low enough to prevent thermal damage. The number of pulses and pulse frequency also play a large part in avoiding thermal damage to the tissue.

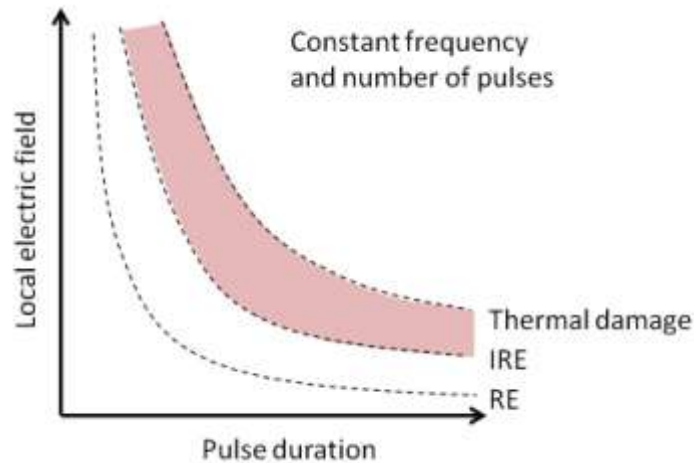


Figure 1.7. Effect of electric field and pulse duration on cell response during electroporation. As can be seen in the illustration, for a constant number of pulses and pulse frequency, increasing the local electric field will allow for shorter pulse duration to obtain the same type of cell response [Pavselj and Miklavcic, 2010]. Possible responses include no effect on the cell, the occurrence of reversible electroporation (RE), the occurrence of irreversible electroporation (IRE) and the combined effect of irreversible electroporation and thermal damage to the tissue. For non-thermal-irreversible electroporation, it is necessary to choose parameters within the colored zone on the figure that result in IRE while keeping below the threshold for thermal damage.

By avoiding thermal damage, irreversible electroporation gains an advantage over other cancer ablation modalities such as cryosurgery and radiofrequency ablation. A major disadvantage of those thermal techniques is that there is a range of temperatures in which cells may survive, resulting in an outer rim around the ablation zone where tissue damage occurs but cell survival is still a possibility. Thus, for treating a cancer tumor, a large buffer region is needed, increasing the ablation zone and resulting in greater damage to the surrounding, non-cancerous tissues. NTIRE, on the other hand, gives an all or nothing result with sharp demarcation between ablated and undamaged cells [Lee et al, 2007]. In addition, by avoiding thermal damage, NTIRE enables the extracellular matrix to remain intact, helping to preserve important structures and functions such as blood vessels and nerves. This, too, is important for cancer treatment, encouraging new cell growth and quick tissue recovery after electroporation treatment.

1.3 MOTIVATION AND DISSERTATION OVERVIEW

1.3.1 Motivation: Non-Thermal Irreversible Electroporation for Cancer Treatment

1.3.1.1 Irreversible Electroporation for Tissue Ablation

The unique method of cell death by irreversible electroporation has been harnessed for ablating cancerous tumors. Irreversible electroporation is viewed to have many advantages over

traditional tumor ablation modalities such as cryosurgery and radiofrequency ablation. First, as mentioned in Section 1.2.9, the well marked ablation zone resulting from irreversible electroporation makes it advantageous, allowing for complete cell death within the ablation zone. Thermal techniques such as radiofrequency ablation are also strongly affected by the blood flow, making the extent of the high temperature treatment area difficult to control [Davalos *et al*, 2005]. In addition, since irreversible electroporation specifically targets the cell membrane, other structures within or adjacent to the tumor may be left intact, allowing for the tissue to recover quickly. The clinical procedure is also relatively fast compared to other cancer ablation methods [Lee *et al*, 2007]. For example, cryoablation not only results in a transition of damage at the lesion margins and injury to adjacent structures such as neurovascular bundles, but clinically applying cryosurgery can be time consuming since multiple freeze-thaw cycles may be needed for cell death to occur [Onik and Rubinsky, 2010]. An additional advantage of irreversible electroporation is that during treatment the affected area can be monitored using electrical impedance tomography [Davalos *et al*, 2005]. Irreversible electroporation is viewed as a cancer treatment method that can be used to address these shortcomings of cryosurgery and other tumor ablation modalities.

Davalos, Mir, and Rubinsky [Davalos *et al*, 2005] hypothesized that irreversible electroporation could be used to ablate substantial volumes of tissue such as cancer tumors in a non-thermal fashion. They demonstrated the feasibility of this technique through mathematical analysis of the thermal aspects of irreversible electroporation on the liver. This work led to a series of experimental studies that demonstrated the ability of irreversible electroporation to be utilized as an ablation method. Miller *et al* [Miller *et al*, 2005] demonstrated that irreversible electroporation could be used to cause complete ablation of hepatocarcinoma cells *in vitro* while avoiding thermal effects. To do this, they used an electric field of 1500 V/cm and 3 sets of 10 pulses each of a 300 μ s length and showed that applying a given amount of energy over a multiple pulse protocol is more effective in cell ablation than applying it in a single pulse. The ability of irreversible electroporation to ablate large volumes of tissue was further demonstrated on an *in vivo* small animal study on the rat liver [Edd *et al*, 2006]. Here, by applying a single pulse of 20 ms at an electric field of 1000 V/cm to the liver, primarily non-thermal damage occurred, and a sharp demarcation between affected and unaffected regions of the tissue was evident around a predicted electric field range of 300-500 V/cm. Larger animal studies also showed that irreversible electroporation can cause liver ablation. Rubinsky *et al*. [Rubinsky *et al*, 2007] applied irreversible electroporation to the pig liver. Not only did they show complete necrotic tissue with the ablated zone, but they also obtained a margin between the ablated and unaffected tissue that was only several cells thick. Complete ablation up to the blood vessels was evident without negatively affecting the artery function, indicating the potential use of irreversible electroporation to treat tumors near large blood vessels. In addition, they demonstrated the use of ultrasound during treatment, using it to position the electrodes in pre-experimentally determined locations based on mathematical analysis as well as to monitor the electroporation progress in real time. Irreversible electroporation was also demonstrated as a tissue ablation modality on the heart in a study on the pig heart's atrial appendages [Lavee *et al*, 2007]. Additional studies have demonstrated the success of irreversible electroporation as a method for treating both benign and malignant tumors in large and small animal models. This includes work includes treating implanted mouse sarcomas [Al-Sakere *et al*, 2007], breast cancer in mice [Neal *et al*, 2010], the prostate [Onik *et al*, 2007], and the brain [Ellis *et al*, 2011].

Irreversible electroporation was also successfully used in a clinical trial, treating 16 patients for prostate cancer [Onik and Rubinsky, 2010]. These patients received treatment on an outpatient basis, and post-treatment biopsies showed no evidence of cancer. Intact flow through the neurovascular bundle was demonstrated immediately after the procedure and all patients remained continent, demonstrating the ability for cancer treatment while preserving the structure and function of the urethra, nerves, and rectum. In addition, irreversible electroporation has been used in clinical trials with success for treating the kidney, resulting in close to complete absence of pain after the procedure, and other researchers have been to apply this ablation modality to the human brain and pancreas [Thomson, 2010]. This work illustrates the success of using irreversible electroporation as an outpatient procedure for tumor ablation, providing a procedure with remarkably quick patient recovery and continued function. The studies described here along with others illustrate that great use of irreversible electroporation for cancer treatment as well as other medical applications.

1.3.1.2 Effect of Irreversible Electroporation on Tissue Recovery and Minimizing Collateral Damage

As described above, irreversible electroporation is a unique method for selective cell ablation that can be used to treat some forms of cancer in place of localized radiation treatments, radiofrequency, and cryoablation. There has been a great deal of research into the effects of radiation therapy on adjacent, normal tissues and the development of methods used to treat potential side effects and collateral damage that can occur [Ciorba and Stenson, 2007; Kountouras and Zavos, 2008; Packey and Ciorba, 2010; Famularo *et al*, 2010; Smith and DeCosse, 1986]. Thus far, however, little has been done to investigate the effects of irreversible electroporation on adjacent tissues. Though the ability of irreversible electroporation to provide focused therapy may make it advantageous in some situations over other cancer treatments such as radiation therapy and cryosurgery, it is nonetheless essential to understand the effects of irreversible electroporation on the surrounding tissues and to investigate how the tissue responds and recovers with time.

This is the main focus of this thesis work. Here, two clinically relevant tissues are chosen that may experience some level of electroporation due to their proximity to cancer tumors. For continued function and recovery after treatment, it is essential that these tissues can survive the electrical protocol and recover quickly. In order to examine how clinically relevant tissues respond and recover with time after receiving irreversible electroporation, the artery and the small intestine were chosen. Both the artery and the small intestine could be adjacent to or embedded within a tumor that may be a potential candidate for irreversible electroporation ablation. Thus, these tissues could experience some of the electroporation effects. Since both the artery and the small intestine are crucial for continued function and recovery, it is essential to know how they recover with time. In addition, current methods for treating abdominal cancer tumors such as chemotherapy and localized radiation treatment will cause damage to the small intestine even though these methods are not directly targeting the small intestine [Han *et al*, 2011; Keefe *et al*, 2000; Ciorba and Stenson, 2009]. The small intestine is a very sensitive tissue, and this potential damage can lead to a great deal of pain and complications for the patient and may even lead to discontinuance of treatment [Keefe *et al*, 2000; Packey and Ciorba, 2010]. Thus, due to the sensitivity of the small intestine, it is very important to see how it responds to irreversible electroporation as a first step in demonstrating the safety of using irreversible

electroporation as an ablation modality for abdominal cancers. This work assesses the ability of both the artery and the small intestine to recover after treatment. It is hypothesized that the extracellular matrix will remain undamaged after treatment and will enable the tissue to sustain new cell growth and recover.

1.3.2 Motivation: Developing Tissue Engineered Tissue Scaffolds with NTIRE

There is a great need for readily available arterial grafts for clinical use such as bypass grafting for the treatment of cardiovascular disease. The use of decellularized tissue as an arterial scaffold is one method that is being developed to meet this need. Common decellularization protocols use a combination of mechanical, chemical, and enzymatic methods to remove the cellular components from the tissue, leaving behind the extracellular matrix (ECM). These methods, however, may cause some damage and changes to the ECM. The ECM's composition and structure is very important not only for the mechanical integrity of the scaffold but also for its ability for cell in-growth and future remodeling. Thus, examining the ability of producing decellularized constructs using NTIRE may provide another method for obtaining a tissue scaffold while maintaining important extracellular components and structure.

1.3.2.1 Motivation for Developing a Decellularized Tissue Scaffold

Tissue engineering attempts to replace diseased tissues of the body with engineered replacements. One of the most important applications of tissue engineering is for treatment of cardiovascular diseases. Clinical treatment of disease and trauma to the coronary arteries and the peripheral vessels often includes the use of bypass grafting. The choice of the graft is critically important and plays a major role in the success of the procedure. Autologous grafts are most often used, and are typically taken from the saphenous vein, internal mammary artery, or the radial artery [Campbell and Campbell, 2007]. This method, however, is not always an option since many patients do not have a vein that is suitable to use. Also, the costs associated with harvesting autologous vessels are considerable, and there is a significant level of morbidity associated with the procedure [Huynh *et al*, 1999].

Synthetic grafts such as Dacron or polytetrafluoroethylene have also been used with some success. When it comes to the treatment of small diameter vessels, however, the use of these grafts tends to lead to poor compliance and low patency, often resulting in thrombogenicity due to lack of endothelial cells and anatomic intimal hyperplasia [Conklin *et al*, 2002]. Thus, an alternative graft is sought that can meet the disadvantages and shortcomings seen in both autologous and synthetic grafts.

Recently, tissue engineering has been looked at as a promising solution to the issues at hand. Such methods often include developing a scaffold that is seeded with cells *in vitro* or implanted and allowed to repopulate *in vivo*. By decellularizing either xenographic or human-based tissue and repopulating it with the recipient's own cells, a scaffold can be derived that, in theory, eliminates the need for immune-suppressant drugs and reduces the risk of graft rejection. Such a scaffold consists of an extracellular matrix (ECM) that is not only rich in cell signaling components essential for cell adhesion, migration, proliferation, and differentiation, but also has a greater resistance to infection than synthetic materials [Yow *et al*, 2006]. Here, the use of an

ECM scaffold in building an arterial graft is examined, and the important structural and functional characteristics of the ECM are briefly reviewed.

1.3.2.2 The Extracellular Matrix as a Tissue Scaffold

The extracellular matrix is a complex structure composed of proteins, glycosaminoglycans, glycoproteins, and small molecules that are secreted by the cells [Martins-Green and Bissel, 1995]. The composition of the ECM varies between different tissue types, and the ECM serves not only to provide structure and strength, but also directly affects the cells, influencing cell attachment, migration, and proliferation, and it has been shown that the elasticity of the ECM can strongly influence the cell phenotype [Engler *et al*, 2006].

Collagen is the most abundant protein in the ECM, and each type of collagen contributes to the distinct mechanical and physical properties of the ECM in each tissue and location throughout the body [Van der Rest and Garrone, 1991]. Type I collagen, for example, is a major structural protein [Van der Rest and Garrone, 1991], and Type IV collagen is found especially in the basement membrane of vascular structures, providing ligand affinity for endothelial cells [Hudson *et al*, 1993]. Fibronectin is also a very important and prominent ECM protein. This dimeric molecule possesses ligands for many different cell adhesions [Miyamoto *et al*, 1998] including the integrin-binding Arg-Gly-Asp (RGD) subunit [Scottile *et al*, 2000] and is critical in the development of vascular constructs. Laminin, an adhesive protein found especially within the basement membrane [Schwarbauer, 1999], is important in the formation and maintenance of the blood vessels. Glycosaminoglycans (GAGs) are also found throughout the ECM and play a crucial role in endothelial cell and smooth muscle cell (SMC) proliferation [Badylak, 2004]. Growth factors found within the ECM are very diverse in structure and function, and are used to modulate cell behavior. These include vascular endothelial cell growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-beta), and platelet derived growth factor (PDGF) [Badylak, 2004]. Proteoglycans are macromolecules that consist of GAGs bound to a protein core [Kjellen and Lindahl, 1991]. They serve a variety of functions such as binding extracellular matrix components, mediating the binding of cells to the matrix, and capturing soluble molecules such as growth factors into the matrix and at cell surfaces [Ruoslahti, 1989].

As can be seen, the ECM is a dynamic and complicated structure that strongly influences the mechanical structure of the tissue, the individual functions of the cells, and the overall tissue's ability to function and remodel. To complicate this even further, important components of the cell and tissue response are mediated by the products of ECM degradation. For example, peptides with antibacterial properties can be released from an enzyme-digested ECM [Sarikaya *et al*, 2002], and the process of digesting the ECM can also result in chemicals that attract progenitor cells [Badylak, 2004].

Common protocols used to build a decellularized scaffold often include the use of xenographic tissue [Huynh *et al*, 1999; Clarke *et al*, 2001; Conklin *et al*, 2002] due to its wide availability. The question often arises as to what potential issues and graft rejection problems might occur from utilizing a scaffold from a different species [Zhang *et al*, 2007]. For example, the terminal galactose alpha 1,3 galactose epitope is expressed on the cell membranes of almost all mammals except humans [Galili, 1993]. Humans and some primates have a natural antibody to this epitope, and xenographic tissue can result in a delayed rejection of the graft [Galili, 1993; Schussler *et al*, 2000]. Removing the cellular components from the tissue prior to implantation,

however, eliminates this issue. Also, since the amino acid sequence and quaternary structure of the ECM components have been shown to be highly conserved across species [Gilbert *et al*, 2006], using a decellularized xenografts provides a means to produce a tissue scaffold that preserves the necessary structural and functional components of the ECM to result in a working blood vessel that can be fully incorporated into the body.

1.3.2.3 Methods Used to Obtain Decellularized Tissue Scaffolds

A variety of decellularization protocols and cell seeding methods have been developed with the goal of building such a graft. Many different protocols have been tested that typically include some combination of physical, chemical, and/or enzymatic processes [Huynh *et al*, 1999; Clarke *et al*, 2001; Conconi *et al*, 2006; Flynn *et al*, 2006]. Physical treatments, such as agitation, mechanical massage, pressure, and freezing and thawing, are used to disrupt the cell membrane and release the cellular content [Gilbert *et al*, 2006]. Enzymatic treatments include the use of trypsin, which cleaves specific peptide bonds [Olsen *et al*, 2004]. Chemical treatments use ionic solutions or detergents such as Triton X-100 [Williams *et al*, 2009; Yazdani *et al*, 2009] and sodium dodecyl sulfate (SDS) [Ott, *et al*, 2008]. These methods all pose some risk of damage to the ECM, possibly compromising the scaffold's further development and integration into the recipient's body [Gilber *et al*, 2006]. For example, chemicals used in the treatment process may not be completely removed after use and could result in long term stenosis *in vivo* due to insufficient cell ingrowth [Conconi *et al*, 2006] or remove important molecules from the collagenous tissue [Gilbert *et al*, 2006]. Physical techniques are also not without potential risk, and can disrupt the ECM as the cellular material is removed [Gilbert *et al*, 2006].

Rosenberg *et al* [Rosenberg *et al*, 1996] were one of the first to implant a decellularized scaffold as an arterial graft, using enzymatic digestion and cross-linking with gluteraldehyde to produce a decellularized bovine arterial scaffold. These grafts were implanted with humans, with seven out of twelve follow-up grafts still patent at 28 months post surgery. The gluteraldehyde cross-linking method, however, makes the graft nonviable and unable to be remodeled by the host [Clarke *et al*, 2001]. Clarke *et al*. [Clarke *et al*, 2001] had greater success, using decellularized bovine uterus as arterial scaffolds that were implanted in dogs and showed fifty percent recellularization within 13 weeks with the presence of smooth muscle cells.

Rosenberg, Clarke, and others were working with large diameter arteries. Small diameter grafts (less than 4-6 mm), however, have proven more difficult to replace, especially in areas of low blood flow, mainly due to the early formation of thrombosis [Zhang *et al*, 2007]. Many groups have looked to solve this problem by modifying the ECM or seeding cells in the scaffolds *ex vivo* prior to implantation [Conklin *et al*, 2002; Borschel *et al*, 2005; Kaushal *et al*, 2001; Yazdani *et al*, 2009]. In addition, Williams *et al*. used TEM and SEM imaging of the ECM collagen fibers and proteoglycans, showing that the macromolecules associated with the ECM not only play a role in cell-matrix interactions but also have a strong effect on the structural integrity of the matrix [Williams *et al*, 2009]. The breadth of this work indicates that a great deal of factors go into developing a decellularized arterial graft. Not only is the structural integrity of the scaffold important from a mechanical point of view, but it is also essential that the scaffold can encourage cell in-growth and function properly once implanted into the body. Studies have shown that this is much more difficult to do for small diameter vessels than for large diameter vessels, and these smaller scaffolds often result in thrombosis when directly implanted. Thus, the procedure used to develop a decellularized graft plays a very important role in how the

scaffold is able to function mechanically and encourage cell in-growth, becoming fully integrated into the body.

1.3.2.4 Potential use of NTIRE to Obtain a Decellularized Tissue Scaffold

Here, NTIRE is utilized to examine the potential of developing a naturally decellularized tissue scaffold that preserves many of the important structural and functional aspects of the extracellular matrix. The motivation for using NTIRE as a scaffold decellularization method came from initial results obtained after examining how NTIRE affects the ability of the artery to regain structure and function after treatment. As discussed within the body of this thesis, artery decellularization occurred. These observations of artery decellularization and recovery with time are examined in further detail within the context of developing a tissue scaffold. Since the use of NTIRE as a tissue scaffold is more of a byproduct rather than the central focus of this thesis, here only an initial investigation into the potential of tissue decellularization is presented. Future work is warranted to further investigate this technique.

1.3.3 Dissertation Overview

This dissertation provides a preliminary assessment of the ability of critical tissues to recovery following an irreversible electroporation treatment protocol. This is especially important to examine for use of irreversible electroporation *in vivo* for tissue ablation purposes. Through pre-experimental analysis and *in-vivo* experiments on small animal models, the recovery process of two clinically relevant tissues is examined: the artery and the small intestine. The main motivation of this work is to assess the recovery process in the case that such critical tissues are adjacent to or embedded within a tumor during irreversible electroporation tumor ablation treatment as well as to gain a further understanding in general of how tissues are affected by irreversible electroporation over time. As an offshoot of this work, the potential of using irreversible electroporation to develop a decellularized arterial construct for tissue engineering purposes is also examined.

Chapter 2 gives a theoretical analysis of the electrical and thermal fields experienced by the artery when an electrical pulse is applied across the artery using plate electrodes. This model corresponds to the experimental procedure in which plate electrodes are used to apply electroporation across the rat carotid artery. This analysis is used to ensure that electrical parameters chosen for experimental testing will minimize any thermal effects to the tissue.

In Chapter 3, the theoretical results obtained for the plate electrode are compared to the thermal and electrical effects modeled for applying irreversible electroporation in a minimally invasive fashion to the artery, using electrodes attached to a catheter. This corresponds to the clinical case in which the artery is being treated with irreversible electroporation directly.

Utilizing the theoretical results from the previous two chapters, Chapter 4 presents the histological results obtained from applying irreversible electroporation directly to the artery *in vivo*. This section follows the recovery of the artery up to one week after electroporation treatment. Based on these experimental results, the artery is seen as a potential tissue for developing a decellularized scaffold. Thus, the implications of these results are discussed both in

context of tissue recovery after electroporation was used for tissue ablation and in context of decellularizing the tissue for tissue engineering applications.

In Chapter 5, pre-experimental modeling gives a prediction of the electrical and thermal effects resulting from applying irreversible electroporation to the small intestine. This analysis uses plate electrodes, corresponding to the experimental procedure in which plate electrodes are used to apply the electroporation treatment directly to the rat small intestine *in vivo*.

In Chapter 6, a more in-depth finite element model of the small intestine is developed. This model incorporates additional complexities such as the heterogeneous tissue layers of the small intestine and anisotropic properties of the muscle layers. The resulting thermal and electric fields indicate that such complexities are important in theoretical analysis.

The theoretical results obtained in Chapter 5 are then used experimentally in small animal survival surgeries, as described in Chapter 7. Here, the recovery of the small intestine after electroporation is examined up to one week after treatment, and regeneration of the small intestine villi is observed within this timeframe. These results are used to assess the safety of irreversible electroporation for abdominal tumor ablation.

Finally, Chapter 8 provides a summary of this dissertation, including important implications from this work and areas of the field that warrant future investigation.

CHAPTER 2: THEORETICAL ANALYSIS OF NTIRE APPLIED TO THE ARTERY

2.1 MOTIVATION AND BACKGROUND

Non-thermal irreversible electroporation (NTIRE) has been developed as a method for controllable cell ablation. Electroporation occurs when an electric field is applied across the cell, destabilizing the electric potential maintained by the cell membrane and resulting in the formation of nanoscale defects in the lipid bilayer. By choosing strong enough electroporation parameters, permanent defects in the cell membrane are created, resulting in cell death from irreversible electroporation. An electric field can, by its very nature, create heating due to the Joule effect. It has been shown, however, that irreversible electroporation can be isolated from this thermal effect, and NTIRE can be used as an independent modality for tissue ablation [Rubinsky, 2007]. NTIRE is unique from other tissue ablation methods. Not only does it avoid thermal damage, but it also produces a well defined region of tissue ablation with sharp, cell-scale borders between the affected and unaffected regions [Lavee *et al*, 2007; Rubinsky *et al*, 2007]. NTIRE specifically targets the cell membrane and thus spares other tissue components such as macromolecules, connective tissue, and the tissue scaffold [Lavee *et al*, 2007].

Recently, the non-thermal controllable cell ablation modality of NTIRE has been harnessed for medical applications such as the treatment of cancer. Miller *et al*. demonstrated the ability of NTIRE to ablate cancer cells *in vitro* [Miller *et al*, 2005], and, in a more recent study, NTIRE was used to successfully ablate the prostate of a dog, demonstrating that structures such as the urethra, vessels, nerves, and the rectum were undamaged by the treatment method [Onik and Rubinsky, 2007]. NTIRE has also shown success in clinical trials for cancer treatment [Onik and Rubinsky, 2010; Thomson, 2010]. The effects of NTIRE on the blood vessels have also been examined for treatment of restenosis, indicating that this technology can be used to quickly and effectively ablate vascular smooth muscle cells (VSMC) without causing damage to the extra-cellular matrix (ECM) [Maor *et al*, 2009; Maor and Rubinsky, 2010; Maor *et al*, 2007]. Understanding how the artery recovers over time after NTIRE treatment is essential. Should an artery be embedded within or adjacent to a tumor, it is important to understand how the artery reacts to the procedure and how quickly it is able to recover in order to ensure continued blood flow and healing to the treated area. In addition, the results of this work indicate that NTIRE may also prove successful as a method for developing a decellularized tissue scaffold for use in tissue engineering applications. Thus, understanding the effect of NTIRE on the artery can lend a greater understanding to both how the tissue responds and recovers *in vivo* as well as the use of this technology in the field of tissue engineering.

One of the key aspects of NTIRE in both cancer ablation applications and in developing a decellularized tissue scaffold for tissue engineering applications is its ability to selectively damage the cell's membrane. Potential Joule heating from the electric field, however, is bound to occur, and cannot be ignored. Though locally induced thermal damage has been utilized with drug delivery for cell ablation applications such as the treatment of cancer [Zhang *et al*, 2009], such heating can also harm the ECM and thus must be avoided here. Previous studies have also examined the effect electroporation and thermal damage on other tissues such as the skin and liver [Becker and Kuznetsov, 2007a; Becker and Kuznetsov, 2007b; Becker and Kuznetsov,

2008; Becker and Kuznetsov, 2007c; Becker and Kuznetsov, 2006]. In order to ensure that NTIRE does not cause significant protein denaturation due to Joule heating effects, the electric parameters must be carefully designed. By decreasing the pulse length and the pulse frequency, the cell membrane can be targeted without resulting in thermal damage to the rest of the tissue components. In order to choose electrical parameters for experimental use that would not cause extensive heating and damage to the tissue, transient finite element analysis of the electrode device was performed, modeling the effect of Joule heating on the temperature distribution of the tissue. These results were then examined to determine the accumulated thermal damage over time and to choose electrical parameters that would minimize that damage.

This chapter examines the electrical and thermal results achieved when applying NTIRE to the artery. The model here is used to analyze what happens when an electrical pulse is applied to a rodent carotid artery. The plate electrode device used in this study is pictured in Figure 2.1. It consists of two printed circuit boards with disk electrodes at the end. The artery is gently pressed between the electrode, and a pulse is applied across the artery.

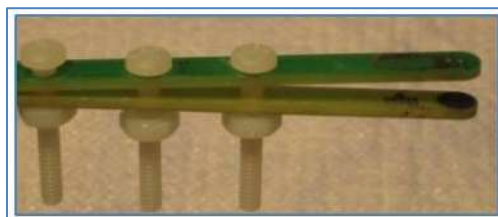


Figure 2.1. Plate electrode used to apply NTIRE. The plate electrode consists of two printed circuit boards with disk electrodes at the end. When used on the rat carotid artery, the electrodes are held apart by approximately 0.4 mm.

2.2 THEORETICAL MODEL OF THE PLATE ELECTRODE DEVICE

Using a finite element program (Comsol Multiphysics 3.5a), the temperature distribution throughout the arterial tissue was modeled. Due to the simplicity of the plate electrode geometry, the artery-plate system was modeled two-dimensionally, as depicted in Figure 2.2. This simplification assumes that the artery and the electrodes are infinite in the axial direction, providing an overestimate of the temperature increase to the artery. The artery's dimensions were based on previous experimental observations, and, since both the electrode plates and the artery are held very close to the body during the procedure, the artery-plate system was modeled as surrounded by air at an elevated temperature of 37 °C. The artery's thermal properties were assumed to be both isotropic and homogenous in cross section (see Fig. 2.2).

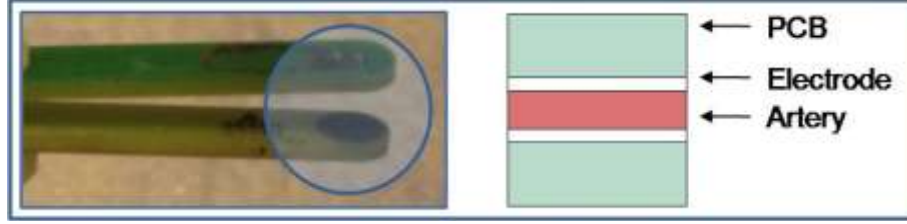


Figure 2.2. Schematic of model geometry for the electrode plate and carotid artery. The artery is shown here in cross-section, pressed between the two electrodes. The artery was modeled as 0.4 mm by 3 mm, and the copper electrodes are 0.1 mm thick. The printed circuit boards were modeled as having the material properties of Flame Retardant 4 (FR4) and have the dimensions of 1.6 mm by 3 mm. The artery-plate system was modeled as being surrounded by a 3 cm by 3 cm block of air.

A solution for a single electroporation pulse was first modeled, using the Laplace equation to evaluate the electric potential distribution.

$$\nabla \cdot (\sigma \nabla \phi) = 0 \quad (2.1)$$

where ϕ is the electric potential and σ is the electrical conductivity. Equation 2.1 can be solved for the heat generation per unit volume (q_{JH}):

$$q_{JH} = \sigma |\nabla \phi|^2 \quad (2.2)$$

The electrodes were represented by a fixed voltage (Dirichlet) boundary condition. For the plate electrode device, the top electrode was set to having a positive potential and the bottom electrode was set to zero:

$$\phi_1 = V_o \quad (2.3)$$

$$\phi_2 = 0 \quad (2.4)$$

where V_o is the potential difference applied across the electrodes during the electroporation pulse. The boundaries between the artery and the air were set as electrically insulating.

Since the artery is exposed to the air during the procedure, the temperature was solved using conduction between the arterial tissue, electrodes, printed circuit boards, and air:

$$\rho C_p \frac{\partial T}{\partial t} - \nabla \cdot (k \nabla T) = q_{JH} \quad (2.5)$$

where ρ is the material density, C_p is the heat capacity, and k is the thermal conductivity. In this model, q_{JH} is determined from the Joule heating and is given in Eq. 2.2. In order to solve for the resulting temperature distribution, the entire system was initially held at the body temperature of the arterial tissue T_o (37 °C). The internal boundaries between the artery, electrodes, printed

circuit board, and air were all defined as thermally continuous, and the edges of the air space were maintained at T_o , providing a conservative overestimate on the rise in temperature to the artery. Thermal constants used in this evaluation are given in Table 2.1.

Table 2.1. Thermal constants used in the simulation. The values obtained for FR4, copper, and air were taken from the COMSOL Multiphysics 3.5a material library.

			Tissue	FR4	Copper	Air
Electrical conductivity	σ	S/m	0.6 [Gabriel <i>et al</i> , 1996]	0.004	5.998×10^7	0.0001
Heat capacity	C_p	J/kg-K	3,750 [Davalos <i>et al</i> , 2003]	1,369	385	1.007×10^{-3}
Density	ρ	kg/m ³	1,000 [Davalos <i>et al</i> , 2003]	1,900	8,700	1.1614
Thermal conductivity	k	W/m-K	0.5 [Davalos <i>et al</i> , 2003]	0.3	400	0.0263
Initial temperature	T_o	°C	37	37	37	37

2.3 THERMAL DAMAGE ANALYSIS

The full procedure utilized N number of square DC pulses of length t_i and a pulse frequency rat of f . The temperature increases during each pulse due to resistive heating. Heat is dissipated due to conduction to the electrodes and surrounding air. By incorporating intervals between pulses where there is no resistive heating, the local rise in tissue temperature is kept to a minimum. In order to solve for the temperature distribution over the course of the procedure for a multiple pulse protocol, MATLAB 2008Rb (version 7.7) was used to run COMSOL Multiphysics 3.5a. A finite-element mesh was incorporated that utilized triangular elements, and the mesh size was varied in order to validate the accuracy of the solution. The coupled electric field and heat transfer equations were solved at each time step after each pulse and after each resting interval, and the transient solution obtained at the end of each time step was used as the initial condition for the next time interval. The maximum arterial tissue temperature was stored directly after the completion of each pulse as well as once every second for three minutes after the last pulse in order to account for the entire thermal damage due to Joule heating effects [Maor and Rubinsky, 2010]. The maximum tissue temperature was used in order ensure that a conservative estimate of thermal damage would be obtained.

Since the thermal damage to biological tissue is dependent on both temperature and time, the Arrhenius equation is often used to quantify these effects [Tropea and Lee, 1992; Lee, 1991; Chang and Nguyen, 2004; Agah *et al*, 1994; Orgill *et al*, 1998; Lee and Astumian, 1996; Wright, 2003]. This model uses Maxwell-Boltzmann statistics to describe how biological molecules at a temperature T are converted from a viable state to a thermally damaged state at a rate K [Lee, 1991]. This reaction can be described by a first-order chemical rate process [Maor *et al*, 2008]:

$$K = A \exp\left(\frac{-E_a}{RT}\right) = \frac{d\Omega}{dt} \quad (2.6)$$

where R is the ideal gas constant, A is a measurement of molecular collision frequency, E_a is the activation energy needed for the molecules to denature, t is time, and Ω is the accumulated damage. The damage parameter, Ω , can be expressed as the logarithm of the relative concentration of the undamaged molecules at time zero and time t_2 :

$$\Omega(\tau) = \ln \left[\frac{C(0)}{C(t_2)} \right] \quad (2.7)$$

The fraction of damaged molecules can be given as:

$$F_d = \frac{C(0) - C(t_2)}{C(0)} = 1 - \exp(-\Omega) \quad (2.8)$$

Where $C(0)$ and $C(t_2)$ are the amount of undamaged molecules at time zero and time t_2 , respectively. From Eq. 2.8, it can be seen that, as an example, $\Omega = 1$ corresponds to 63.2% of the arterial tissue molecules having reached a thermally damaged state [Diller and Pearce, 1999]. The Arrhenius equation given in Eq. 2.6 can be used to calculate the Henriques and Moritz thermal damage integral:

$$\Omega(t) = \int A \exp\left(\frac{-E_a}{RT}\right) dt \quad (2.9)$$

The values of A and E_a are based on experimental data and depend on the type of tissue under consideration [Diller and Pearce, 1999]. Wright *et al.* [Wright, 2003] showed that the activation energy (E_a) and the natural log of the frequency factor (A) can be plotted for a variety of mammalian protein and tissues values from literature to obtain a straight line correlation given in the following equation:

$$E_a = 2610 \ln(A) + 26200 \quad (2.10)$$

For this analysis, the activation energy was taken from a previous study [Pearce and Thomsen, 1992] where E_a was determined for arterial tissue, and Wright's correlation was used to estimate the corresponding value of A . These values are listed in Table 2.2. Equation 2.9 was applied to the entire procedure. By utilizing the maximum tissue temperature at each time step, an upper bound on the potential thermal damage to the tissue was obtained.

Table 2.2. Constants used in the Arrhenius equation for arterial tissue.

Frequency factor	A	1/s	1.552×10^{67}	
Activation energy	E_a	J/mol	430,000	[Agah <i>et al</i> , 1994]
Ideal gas constant	R	J/mol-K	8.314	[Agah <i>et al</i> , 1994]

2.4 ELECTRICAL PARAMETERS MODELED

For the clamp electrode, the electric parameters that were analyzed by this model were determined from previous experiments [Maor *et al*, 2009] to produce NTIRE in arterial tissue. These parameters consisted of 90 pulses of 70 V (corresponding to a 1,750 V/cm electric field). Each pulse was 100 μ s in length and the pulse frequency was either 1 Hz or 4 Hz. These parameters are summarized in Table 2.3.

Table 2.3 Electric parameters analyzed.

Parameter			
Applied voltage	V_o	V	70
Electric field	--	V/cm	1,750
Pulse length	t_1	μ s	100
Number of pulses	N		90
Frequency	f	Hz	1 Hz or 4 Hz

2.5 RESULTS

As can be seen in Figure 2.4, due to the flat, parallel-plate geometry, applying an electrical potential of 70 V to the tissue results in a uniform electric field throughout the tissue of 1750 V/cm.

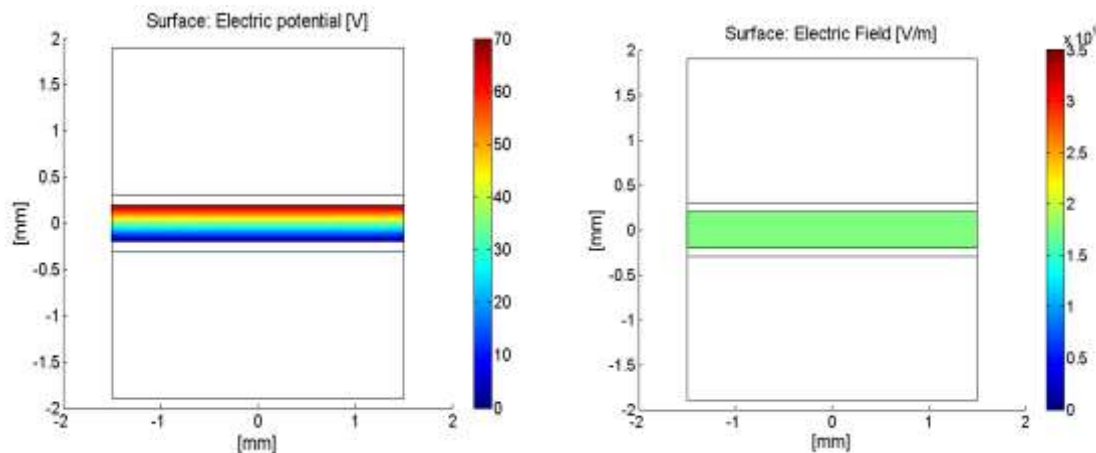


Figure 2.4. The electric potential and resulting electric field experienced by the tissue model.

For a 1 Hz frequency, the overall maximum temperature obtained from the simulation was 316.71K (43.56 °C) corresponding to the maximum temperature of the tissue immediately after the 90th pulse was applied. For a 4 Hz frequency, the overall maximum temperature was

318.4 K (45.25°C). The maximum temperatures were recorded after each pulse and during the three minute cooling period after the pulses were applied, and these are shown in Figure 2.5. In many sources, 315.15 K (42 °C) is taken as the onset of thermal damage [Tropea and Lee, 1992; Dickson and Calderwood, 1980], and it can be seen in Figure 2.5 that the temperature for both frequencies exceeds this threshold during pulsing.

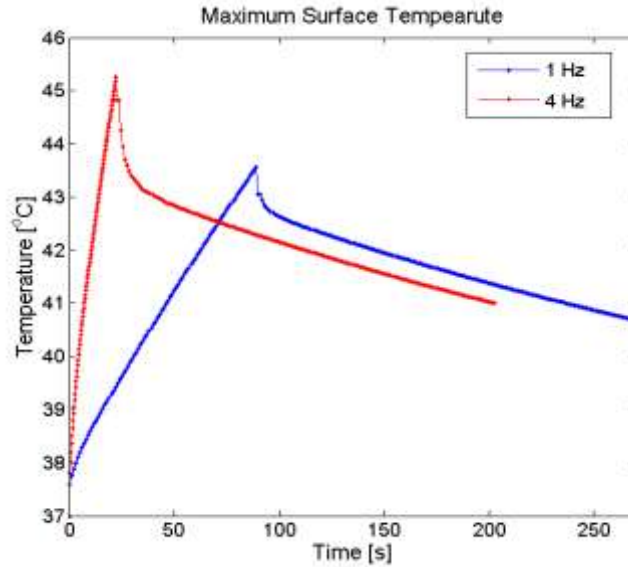


Figure 2.5. Maximum temperatures obtained over the course of the simulation. The maximum temperatures obtained at time steps throughout the simulations show a peak maximum temperature obtained after the final electric pulse followed by a cooling down period for a 1 Hz frequency (blue) and a 4 Hz frequency (red).

Thermal damage, however, is due not only to temperature but also to how long it is applied to the tissue. In Figure 2.5, the maximum temperature is only seen immediately after each pulse. In between each pulse, however, the tissue is able to cool due to conduction to the electrodes and the surroundings. Using the Henriques and Moritz thermal damage integral (Eq. 2.10), a better estimate of the thermal damage over the entire heating and cooling phases was quantified, giving a value of $\Omega = 0.0188$ for a 1 Hz frequency, corresponding to 1.86% damage, and $\Omega = 0.0199$ for a 4 Hz frequency, corresponding to 1.97% damage.

2.6 DISCUSSION AND CONCLUSIONS

Here, a simple finite element model is used to demonstrate that the electrical parameters chosen for NTIRE of the carotid artery will not produce thermal damage. Thermal damage due to Joule heating is an undesired effect for many electroporation applications such as cancer treatment and the development of a tissue engineered scaffold. The main goal here of electroporation for cancer treatment would be to ablate the cancer cells while leaving the artery

intact and allowing for quick recovery. For tissue engineering applications, the goal is to selectively ablate the cells within the tissue while preserving the cell scaffold. In both cases, it is important that the scaffold is not damaged by the procedure, allowing either for quick recovery *in vivo* or for encouraging new cell growth and artery functionality. Heating to the scaffold could result in damage which would make it harder or even impossible for the scaffold to encourage cell growth after treatment and to continue proper function. Mathematical models of Joule heating are very useful tools that can be quickly used to predict tissue temperatures throughout the NTIRE treatment procedure.

This chapter examines the thermal effects of electrical parameters that have been shown to be strong enough to cause electroporation cell ablation to the vascular tissue. The model used here incorporates a very simplified geometry as well as boundary conditions that are used to over predict any thermal damage that might occur. This over prediction is used to ensure that any Joule heating to the tissue *in vivo* will remain well below the threshold for thermal damage and extracellular matrix destruction. Heat losses from the tissue due to both convection and radiation have been neglected in this analysis. Leaving out the effects of convection incorporates an additional factor of safety into the analysis. Biological tissue properties can vary greatly from one source to another, and thermal and electrical properties are not available for at all levels of the tissue organization. For example, though properties were found in the literature for bulk arterial tissue, properties for each layer of the artery are lacking. By modeling the tissue as homogenous, we obtain a constant electric field throughout, as shown in Figure 2.4. It is, however, highly likely that the electrical conductivity, thermal conductivity, density, and specific heat varies from the adventitia layer to the medial layer to the endothelial layer as well as within each layer due to the different composition of each layer. For this study, however, such detailed modeling is not necessary. The exact amount of heating to the tissue does not need to be known. Rather, it is important only to ensure that any heating resulting from the choice of electrical parameters does not result in damage to the tissue. Thus, including simplifications in the model that result in a higher temperature prediction ensure that the chosen parameters will not cause thermal damage.

Here, it was seen from the finite element analysis that, for the electrical parameters modeled, both a 1 Hz and a 4 Hz protocol result in less than 2% thermal damage. As mentioned previously, many sources cite 42 °C as a threshold for thermal damage. These changes to the tissue due to heating, however, depend on both the time for exposure as well as temperature. Although both electrical parameter protocols result in maximum temperatures that briefly exceed this threshold, both protocols can be shown to result in minimal thermal damage when taking both temperature and time into account with the Arrhenius equation. Also, it must be emphasized that this study examines the maximum temperature seen throughout the tissue. Thus, only a very small portion of the tissue model actually gets anywhere near a 2% level of damage. The rest of the tissue experiences a far lower level of damage. This model, therefore, indicates that the electrical parameters chosen for NTIRE treatment of the rat carotid artery will be safe to use *in vivo*, and it can be assumed from this model that any resulting cell ablation occurs due to electroporation and not due to thermal heating effects.

Finite element models go hand-in-hand with electroporation, illustrating that thermal damage can be easily avoided with the right combination of electrical properties. Though the carotid artery model shown here is very simple due to the geometry of the plate electrodes and

simplifying assumptions, more complicated geometries can be easily analyzed using the same basic equations and methodologies detailed here.

CHAPTER 3: COMPARING THE THEORETICAL ELECTRICAL AND THERMAL EFFECTS OF TWO DIFFERENT ELECTRODE DEVICES

3.1 MOTIVATION AND BACKGROUND

Previous work by Maor *et al* [Maor *et al*, 2010] examined the effects of thermal damage to the arterial tissue when irreversible electroporation was applied through an endovascular device in a minimally invasive manner. The endovascular device is pictured in Figure 3.1, and consists of four electrodes made of rectangular nickel titanium wire, an electrically insulated catheter shaft, and a standard polyethylene terephthalate non-compliant balloon. The electrodes are oriented parallel to the catheter shaft and over the balloon, and they are spaced out evenly around the circumference of the balloon. The electrodes can be retracted into a flexible tube in order for the device to be maneuvered to the desired artery location. Once in place, the electrodes can be expanded by pushing them forward out of the tube and gently pressed in contact with the inner wall of the artery by balloon inflation.

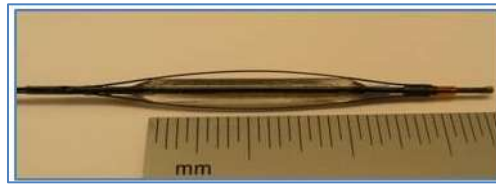


Figure 3.1. Endovascular electrode used to apply NTIRE. The electrode catheter is shown in its inflated state. When in use, the four electrodes are pressed gently against the inner wall of the artery.

Here, the electrical and thermal effects of applying NTIRE through the endovascular device are compared to those obtained by apply NTIRE to the artery using the plate electrodes (described in Chapter 2). Comparing these two methods of arterial ablation with NTIRE is important both theoretically and experimentally (Chapter 4). Using the plate electrode technique is a straightforward method for applying NTIRE directly to the artery in the lab setting in order to investigate how a given electric field affects the artery's ability to recover over time. The endovascular device, on the other hand, may be more clinically relevant for cases in which it is desirable to apply NTIRE directly to the artery. This method, however, requires a more costly and complicated procedure and may not be as well suited for the lab setting. Thus, it is important to understand if the two different methods of applying NTIRE to the arterial tissue will result in similar results in terms of recovery. As a first step, the theoretical models for electric fields and thermal effects are compared, helping to choose electrical parameters for each electrode device that will provide sufficient irreversible electroporation effects to the tissue while minimizing thermal damage.

3.2 THEORETICAL MODEL OF THE ENDOVASCULAR ELECTRODE DEVICE

The endovascular device uses four longitudinal electrodes in contact with the inner surface of the arterial wall. A detailed description of the endovascular device was published elsewhere [Maor and Rubinsky, 2010]. In a manner similar to that of the clamp electrode device, this system was reduced to a two-dimensional model. The inner diameter of the artery was taken as 2.5 mm, based on an average diameter of rabbit iliac arteries. Since this models an intravascular procedure, the artery was assumed to be embedded in a large block of tissue.. This two-dimensional model (depicted in Figure 2) assumes that the electrodes are infinite in the axial direction, providing an overestimate on the resulting tissue temperature since in reality the electrodes are insulated on their ends and only contact the artery over 2 cm of their length.

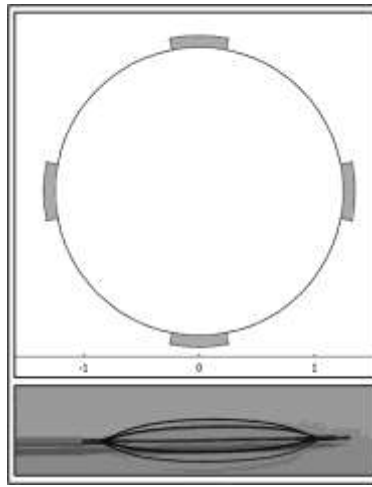


Figure 3.2. Two-dimensional geometry for the endovascular device. The four electrode nickel titanium wire electrodes (0.5 mm x 0.4 mm in cross-section) run parallel to the longitudinal axis of the artery and lay pressed against the inner artery wall (2.5 mm in diameter). The electrodes are insulated from the arterial lumen space, and the whole construct is modeled as being embedded in a very large block of tissue (not shown in full). Dimensions shown here are in millimeters.

The electrical pulse is modeled in a manner similar to that of the clamp electrode device, using the Laplace equation as given in Equations 2.1 and 2.2. The electrodes utilize a bipolar design with two electrodes having a positive potential and two electrodes having a potential of zero. All boundaries of the system not in contact with the electrodes were assumed to have a zero electric flux boundary condition:

$$\frac{\partial \phi}{\partial n} = 0 \quad (3.1)$$

Since the artery is assumed to be embedded within the tissue, the Pennes bio-heat equation was used to determine the temperature distribution:

$$\nabla(k_t \nabla T) + \omega_b c_b (T_a - T) + q_{JH} + q + \sigma |\nabla \phi|^2 = \rho_t c_{p,t} \frac{\partial T}{\partial t} \quad (3.2)$$

where k_t is the thermal conductivity of the tissue, T is the temperature, ω_b is the blood perfusion rate, c_b is the heat capacity of the blood, T_a is the arterial tissue temperature, q_{JH} is heat generation obtained from the Joule heating (Eq. 2.2), q is the basal metabolic heat generation, ρ_t is the tissue density, and $c_{p,t}$ is the tissue heat capacity. It was assumed that the metabolic heat source was insignificant [Davalos *et al*, 2003]. The initial temperature of the entire domain was set at the physiologic arterial tissue temperature (T_a). The boundaries along the inner surface of the artery were taken to be adiabatic in order to predict maximal temperature rise along the arterial wall. The outer boundary of the large block of tissue was held at constant physiological temperature (T_a) throughout the simulation. The thermal and biological properties used in this analysis are given in Table 3.1.

Table 3.1. Thermal and biological constants used in the simulation. The thermal and electrical properties were obtained from [Gabriel *et al*, 1996; Davalos *et al*, 2003; Lee and Despa, 2005; Wissler, 1998].

			Tissue	Blood	Catheter electrode
Electrical conductivity	σ	S/m	0.6	--	4.032×10^6
Heat capacity	C	J/kg-K	3,750	3,640	100
Density	ρ	kg/m ³	1,000	1,000	--
Thermal conductivity	k	W/m-K	0.5	--	--
Perfusion rate	ω	1/s	--	0.0005	--
Tissue temperature	T_a	°C	37		

Heat is dissipated due to conduction to the surrounding tissue for this catheter electrode design.

The electric parameters used for the endovascular electrodes consisted of 90 pulses of 100 μ s in length and a pulse frequency of 4 Hz. A voltage of 600 V was used, corresponding to an electrical field of 1,000 V/cm or higher. Heating effects were determined as described previously in Chapter 2 using Equation 11. Values for the Arrhenius equation used an activation energy of $E_a = 430,000$ J/mol and a frequency factor of $A = 5.6 \times 10^{63} \text{ s}^{-1}$ [Agah *et al*, 1994].

3.3 THEORETICAL MODEL OF THE PLATE ELECTRODE DEVICE FOR COMPARISON

In order to compare the thermal and electrical effect obtained for the plate electrode to those previously obtained for the endovascular electrode design, the plate electrode was modeled as described previously (Chapter 2). Some changes in the electrical and thermal damage parameters, however, were utilized in order to enable a direct comparison between the

endovascular device and the plate electrodes. Thus, the plate electrodes were modeled as having 90 pulses of 100 μ s length at a 4 Hz frequency. A 70 V potential was applied across the plates, resulting in an electric field of 1,750 V/cm. The electrical conductivity of the tissue was changed to 0.6 S/m in order to better compare with the results obtained for the endovascular device, and to enable for a more conservative estimate of the resulting thermal damage. The same Arrhenius equation parameters as used for the endovascular device were used here for comparison purposes, incorporating an activation energy of 430,000 J/mol and a frequency factor of $5.6 \times 10^{63} \text{ s}^{-1}$. Once again, thermal damage was determined using Equation 2.9.

3.4 RESULTS

Here, the results obtained from the plate electrode with a 4 Hz frequency and a tissue electrical conductivity of 0.6 S/m are compared to the thermal analysis obtained from the catheter electrode. The solution to the Laplace equation for the electric potential distribution is static and independent of time. For each applied pulse, the electric field is non-transient. The electric field obtained from the clamp electrode is constant over the entire artery at 1,750 V/cm due to the simple geometry. The electric field distribution for the catheter electrode design is shown in Figure 3.3.

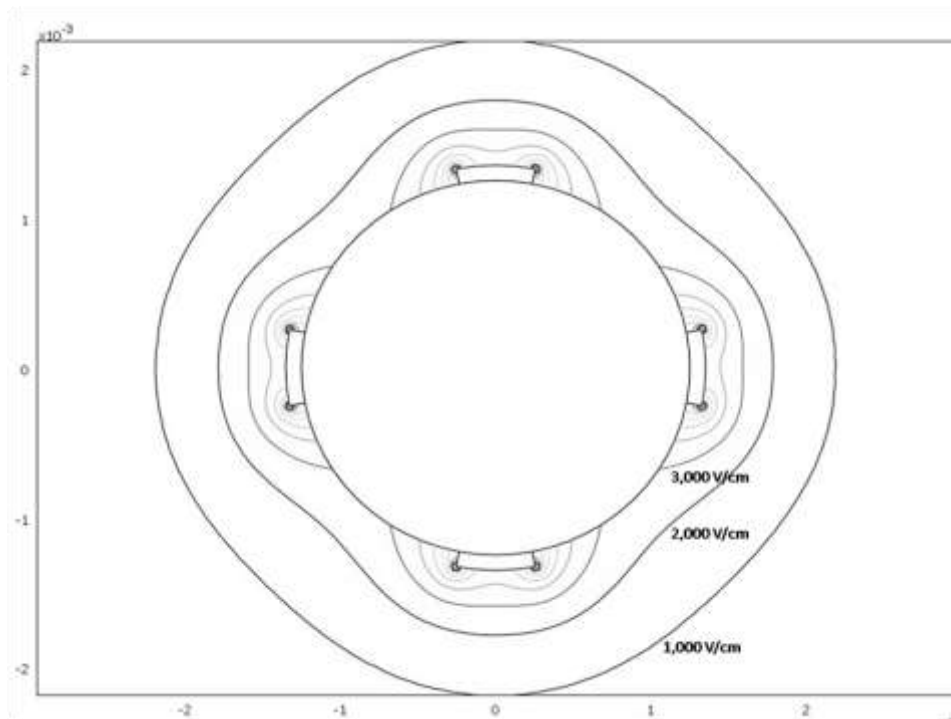


Figure 3.3. Two-dimensional electric field distribution. The resulting electric field is shown for the catheter electrode device. The outermost contour corresponds to 1000 V/cm, and the electric field increases by 1000 V/cm for each contour moving in towards the electrodes. A spike in the electric field is seen at the corner of the electrodes due to edge effects. The model dimensions are shown in meters.

The maximum temperature obtained for each model was recorded after each pulse and during the simulated cool down period following the applied pulse procedure. These results are shown in Figure 3.4. The overall maximum temperature for the plate electrode device obtained from the simulation was 45.25°C. The electric parameters applied to the endovascular electrode device induced a maximum temperature of 66.8 °C. The maximum tissue temperature was obtained immediately after the 90th pulse for both electrode designs.

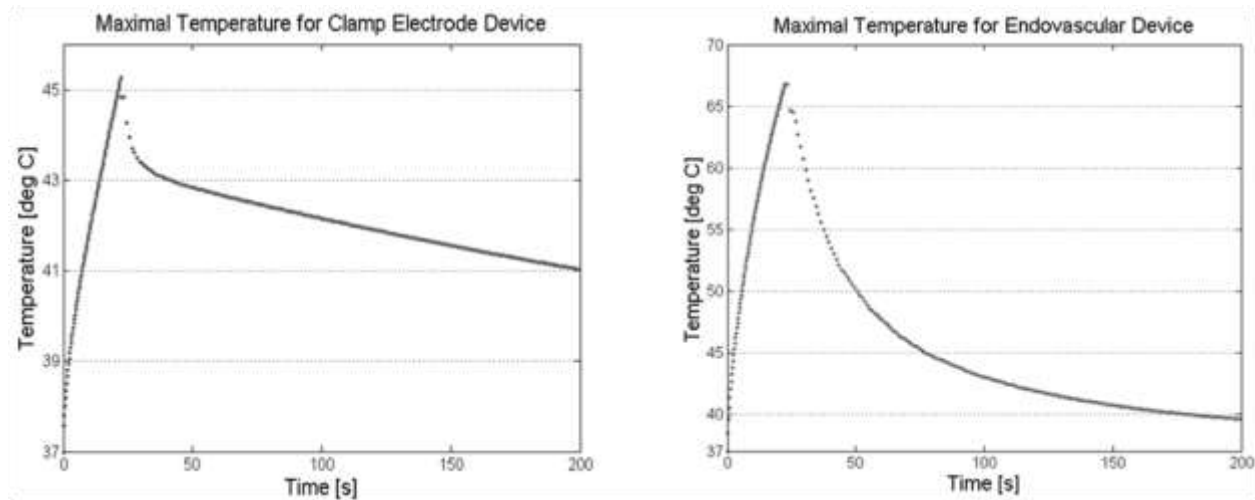


Figure 3.4. Transient solution of the maximum tissue temperature. The maximum temperature obtained for each time step over the course of the simulation is plotted for the plate electrode design (left), indicating that the overall peak temperature is reached immediately after the final electrical pulse, as expected. The maximum temperature obtained for the first 200 μm of the biological tissue domain are shown for the endovascular device (right).

The Arrhenius damage integral (Equation 2.9) was evaluated to quantify the thermal damage obtained over the entire heating and cooling phases. This gave a value of $\Omega = 7.163 \times 10^{-6}$ for the plate electrode design, corresponding to negligible damage to the molecules due to Joule heating effects. The endovascular design resulted in $\Omega = 0.0159$ indicating that approximately 1.6% of the molecules in the areas of maximal temperature became thermally damaged.

3.5 DISCUSSION AND CONCLUSIONS

Thermal damage is eliminated by controlling the electrical parameters and minimizing Joule heating. Mathematical modeling of the effect of these electrical parameters using the Arrhenius equation gave thermal damage values of $\Omega = 7.163 \times 10^{-6}$ and $\Omega = 0.0159$ for the plate electrode device and the endovascular device, respectively. This represents only 1.6% damage of the tissue molecules for the larger thermal damage case. This estimate gave an upper bound on potential tissue damage due to Joule heating since it utilized the maximum temperature seen

throughout the tissue over the entire course of the simulation. Further efforts were made so that this model would over-predict the amount of thermal damage. For example, both electrode devices were modeled as being two-dimensional, resulting in an assumption that an electrode pulse was being applied along an infinite length of the artery when, in reality, the electrical pulse was only applied along 0.5-2 cm of the artery's axis. Also, when modeling the electrode clamp device, only conduction between the artery, electrode clamp, and air were considered as a cooling method, ignoring any heat loss due to natural convection. The electrical and thermal model of the endovascular device did not incorporate heat convection due to the adjacent vein, and the tissue conductivity used in both models was taken as 0.6 S/m.

NTIRE is a very simple and controllable cell ablation technology. This has been shown from previous studies for the treatment of cancer [Miller *et al*, 2005; Onik and Rubinsky, 2007]. Not only can electrical parameters be chosen such that thermal damage is avoided, but the electrical parameters and electrodes can also be designed in such a way as to control the electric field and thus the extent of cell ablation. The clamp electrode device, as modeled here, results in an electrical field of 1,750 V/cm between the two electrodes. Cells ablated using this device must be contained in the tissue placed between the two electrodes. This electric field can be controlled further using more complex electrode geometry, as demonstrated for the endovascular electrode device. Previous studies have shown that, when using 90 electric pulses, an electric field of 1,750 V/cm is required for successful arterial cell ablation [Maor *et al*, 2009]. As shown here, the resulting electrical field can easily be modeled even when, as with the endovascular electrode device, the electric field varies spatially. As illustrated in Figure 3.3, the electric field, and hence the extent of cell ablation, can easily be visualized.

The clamp electrode and the endovascular electrode device apply NTIRE to the artery utilizing different methods, resulting in advantages and disadvantages to both techniques. The clamp electrode utilizes a uniform electric field between the electrodes. As a result, all tissue within the ablation region experiences the same electric parameters. The endovascular device, on the other hand, results in an electric field profile that decreases with distance from the electrodes and that spikes around the corners of the electrodes as seen in Figure 3.3. Finite element modeling also indicates that using the clamp electrode results in much less thermal damage than seen with the endovascular device. Nonetheless, though the endovascular device may result in greater thermal damage and a varying electric field, it still performs well. The electric field may vary due to the endovascular device's more complicated geometry, but the device is still very simple to model and results in NTIRE that is very controllable. From the finite element modeling, it was determined that the endovascular device results in only 1.6% molecular denaturation at the corners of the electrodes where points of maximum temperature occur. All other areas of the tissue would experience much less thermal damage. Also, though the electric field varies with the distance from the electrodes, NTIRE is unique in that it either results in cell death or leaves the cells undamaged. Thus, by knowing the electric field necessary to induce electroporation, the area of cell ablation can be easily predicted. The endovascular technique described here allows for tissue ablation utilizing minimally invasive methods, reducing the risk of pain, infection, and other complications that can be experienced with the open surgery needed to apply NTIRE using the electrode clamp device. As can be seen, though both methods have their own advantages, the endovascular technique may become the preferred option due to its minimally invasive characteristics.

CHAPTER 4: NTIRE RESULTS IN ARTERY DECELLULARIZATION IN VIVO

4.1 MOTIVATION AND BACKGROUND

4.1.1 Motivation for Cancer Treatment

Irreversible electroporation has shown success as a new minimally invasive surgical technique used to treat biological tissues for cancer treatment and other applications where controlled tissue ablation is warranted. By controlling the electrical parameters, electroporation can be harnessed to specifically target the cell membrane, causing cell death by pore formation while theoretically avoiding any thermal damage to the surrounding tissue structure by Joule heating [Davalos *et al*, 2005]. Non-thermal irreversible electroporation (NTIRE) is often advantageous over other cellular ablation treatments in that it is able to directly target cells and results in either cell death or cell survival, with a very well marked transition zone between ablated and un-ablated tissue of only a few cell thick [Rubinsky *et al*, 2007]. Though this biophysical phenomenon is not completely understood [Teissie *et al*, 2005], it has nonetheless found extensive use in the field of medicine. In addition, NTIRE can be applied *in vivo* in a minimally invasive manner, further increasing its clinical appeal. Indeed, NTIRE has shown success in clinical trials for the treatment of prostate cancer [Onik and Rubinsky, 2010] as well as the kidney [Thomson, 2010].

Despite the success of NTIRE both experimentally and in clinical trials for cancer ablation, there have been no systemic studies on how normal critical tissues near the ablation site respond and recover over time after treatment. The artery is one such that warrants further investigation. Often, a tumor may be adjacent to an artery. In cancer treatment, it is essential that complete tumor ablation occurs, but it is also important that the artery is able to continue functioning, helping the treated area to heal and recover quickly. Thus, knowledge of how NTIRE specifically affects the artery is vital for further developing this cell ablation technique for additional cancer treatment applications as well as other medical purposes.

Here, small animal experiments are used to examine the recovery of the artery after NTIRE. The artery is examined up to one week after applying NTIRE directly to the artery, and histological analysis is used to assess how the tissue is affected and recovers with time. The results of this work are important not only for treating tumors near important arteries, but also for providing more knowledge to the field of electroporation that may be beneficial in developing additional medical applications for NTIRE.

4.1.2 Motivation for Tissue Engineering Applications

Arterial grafts are very important in treating cardiovascular disease through bypass grafting. According to the American Heart Association, approximately 448,000 cardiac revascularizations are performed on a yearly basis in the United States alone. Developing tissue engineered grafts is one solution to meet the shortcomings in using autologous or synthetic grafts.

A variety of methods have been employed to develop tissue engineered grafts for the replacement of diseased or damaged tissues and organs. Some of these methods have focused on developing a scaffold that is either seeded with cells *in vitro* or directly implanted and allowed to repopulate *in vivo*. Though a great deal of research has been aimed at developing biodegradable polymer scaffolds, others have focused on producing natural scaffolds for developing such tissue engineered grafts. Such a natural scaffold can be produced by decellularizing xenographic or human based tissue and repopulating it with the recipient's own cells, eliminating the need for immune-suppressant drugs and reducing the risk of graft rejection. Most tissue decellularization methods typically include some combination of physical, chemical, or enzymatic processes [Huynh *et al*, 1999; Clark *et al*, 2001; Conconi *et al*, 2006; Flynn *et al*, 2006]. Though these have shown promise, as demonstrated by the work of Ott *et al*. [Ott *et al*, 2008] in developing a decellularized heart, there has, in general, been little long term follow-up [Campbell and Campbell, 2007], and some of the methods commonly employed been shown to potentially risk damage to the ECM [Gilbert *et al*, 2006].

Here, a method for tissue decellularization is examined that utilizes non-thermal irreversible electroporation (NTIRE) and the body's host response, possibly including immunological mechanisms. Recently, the effect of NTIRE on blood vessels has been investigated for use in the treatment of restenosis [Maor *et al*, 2009; Maor *et al*, 2008; Maor *et al*, 2007]. Maor *et al*. [Maor *et al*, 2009] has shown that NTIRE can ablate VSMC within seconds without causing damage to the extra-cellular components, demonstrating a possible treatment method for restenosis. It is hypothesized that the strength of NTIRE can be ideally suited for the development of a decellularized tissue scaffold.

There exists a large potential for the development of several methods that use NTIRE to derive a decellularized tissue scaffold, with the most straightforward being to simply apply NTIRE to the xenographic or a human donor tissue just prior to implantation. The simplicity of this method is substantially advantageous, and it may become the method of choice. However, the immune response and other cellular and enzymatic processes involved in the removal of the dead cells by the host organism may prove detrimental to the host. Since there has been very little research on the immunological response to NTIRE cell damage [Rubinsky *et al*, 2007; Al-Sakere *et al*, 2007], substantial work remains before this method can be applied. Another method, inspired by previous observations, may be more immediately applicable. It involves applying NTIRE to the donor tissue, waiting for the donor's host response to depopulate the cells, and then harvesting the tissue scaffold that has remained. The decellularized construct would then be implanted into the recipient, and the cells would be allowed to repopulate *in vivo*.

4.1.3 Goal of Study

This study has implications for two different areas of investigation:

1. Safety and artery recovery after NTIRE ablation for applications such as cancer treatment.
2. Development of a decellularized tissue construct for applications in the field of tissue engineering.

The goal of this study is to examine how the artery recovers after receiving NTIRE-treatment. From a recovery and safety aspect for cancer ablation, the focus will be on how the extracellular matrix is preserved and how quickly new cells are able to grow. For the application of tissue decellularization, the observations on artery recovery are expanded upon. Here, the goal is to characterize the following attributes of the proposed decellularization process: a) apply NTIRE to the selected tissue, b) determine the area of decellularization as well as when decellularization is complete, and c) determine if the decellularized scaffold can regain function as measured by re-endothelialization of the vessel lumen. An additional focus is placed on applying the cell ablation methodology and obtaining a decellularized arterial scaffold using two different methods: applying the electrodes to the outside of the artery and applying the electrical field minimally invasively using endovascular electrodes as described in Chapter 3.

4.2 METHODS

The experimental protocol used here follows that used by Maor *et al.* [Maor *et al.*, 2009] to ablate blood vessel cells with NTIRE for the treatment of restenosis. Fifteen Sprague-Dawley rats weighing 200-300 grams were used in this study. All animals received humane care from properly trained professionals in compliance with both the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (NIH publication No. 85-23, revised 1985).

Animals were anesthetized with an intramuscular injection of ketamine and xylazine (90 mg/kg and 10 mg/kg, respectively), and anesthesia was administered throughout the procedure with vaporized isoflurane. The left common carotid artery of each animal was exposed and a custom-made electrode clamp, as described previously [Maor *et al.*, 2008] and illustrated in Figure 2.1, was applied very close to the carotid artery's bifurcation. The measured distance between the electrodes was approximately 0.4 mm. A sequence of 90 dc pulses of 70 V (corresponding to an electric field of approximately 1,750 V/cm), 100 μ s each, and a frequency of 1 Hz or 4 Hz was applied between the electrodes using a high voltage pulse generator designed for electroporation procedures (ECM 80, Harvard Apparatus, Holliston, MA). These parameters were chosen due to their ability to produce irreversible electroporation without causing thermal damage, as shown in previous work [Maor *et al.*, 2009] and by computer modeling (as discussed in Chapter 2). The procedure was repeated in two successive locations along the common carotid artery, treating approximately 1.5 cm along the length. The right common carotid artery was left alone and used as a control. The animals were divided into five groups. The first three groups utilized a 1 Hz frequency and the animals were kept alive for three, five, and seven days respectively prior to being euthanized. The fourth and fifth groups incorporated a 4 Hz frequency in the electrical parameters, and the animals were kept alive for either three or seven days prior to being euthanized.

In order to experimentally test the endovascular electrode device for comparison with the plate electrode device, the iliac artery of New-Zealand white rabbits was chosen as the model for this study since its dimensions are similar to that of the human coronary artery. Here, the work of Maor *et al.* [Maor *et al.*, 2010] is briefly described. The use of these animals was approved by the Institutional Animal Care and Use Committee of ISIS services facility in Berkeley. Anesthesia was induced by ketamine (35 mg/kg) and xylazine (5 mg/kg), and this was followed

by endotracheal intubation and isoflurane for anesthesia maintenance. Sterile techniques were used throughout the procedure. A 4F introducer was placed in the right femoral artery, the endovascular device was inserted in a retrograde manner, and angiography guidance was used to advance the catheter to the aortic bifurcation. The endovascular NTIRE device was inflated along the first two centimeters of the right iliac artery, and an electrical sequence of 90 pulses of 600 V, 100 μ s length, and a 4 Hz frequency was applied using a high voltage pulse generator (ECM 830, Harvard Apparatus, Holliston, MA). The endovascular NTIRE device was then removed, and control angiography was performed to confirm patency of the vessel. The iliac artery was ligated, and the surgical wound was sutured closed. Animals recovered and were housed in the animal facility for seven days prior to being euthanized.

For both experimental groups, animals were anesthetized with ketamine and xylazine prior to being euthanized by an overdose of isoflurane and a bilateral chest dissection. The arterial tree was perfused with 10% buffered formalin. One and a half centimeters of both the left and right carotid artery from the rat groups and 3 cm segments of both iliac arteries from the rabbit groups were harvested, fixed in formalin, and submitted to independent pathology labs (Charles River Laboratories Pathology Associates, Frederick, MD and Pathology Associates, Inc., Berkeley, CA). For the rat carotid artery, three samples from the 3-day, 1 Hz group and four samples from the 5-day, 1 Hz group were cut longitudinally along the length of the artery, and all other samples were cut perpendicular to the axis, exposing the artery's cross-section. Each sample was stained with hematoxylin and eosin (H&E). Select samples from each group were cut in cross-section and were stained with elastic Van Gieson (EVG), Movat's pentachrome stain, or Masson's trichrome in order to examine the integrity of the ECM. DAPI staining was used to detect the presence of DNA. Samples were also selected for use in immunohistochemical analysis using specific antibodies (HistoTec Laboratories, Hayward, CA) for α -smooth muscle actin (α -SMA) and Factor VIII in order to detect the presence of vascular smooth muscle cells and endothelial cells, respectively. The rabbit carotid artery samples were stained with hematoxylin and eosin (H&E), and select samples were stained with Masson's trichrome and elastic Van Gieson (EVG) in order to determine the ability of NTIRE to ablate the vascular cells and the effect of the ablation method on the ECM, particularly the collagen and elastin fibers.

Examination of each section for three, five, and seven days was focused on the effect of NTIRE on the cells in the tunica media as well as the endothelial layer. The structure of the ECM for treated arteries was compared to that of non-treated arteries to ensure that the ECM was not damaged by the applied electric pulse.

4.3 PHYSIOLOGICAL RESULTS

4.3.1 Rat Carotid Artery using Plate Electrodes

Histological analysis of the rat carotid artery three, five, and seven days after being treated with NTIRE with a 1 Hz frequency was used to compare the NTIRE-treated group and the control group. Compared with the control, successful NTIRE resulted in an artery that was largely decellularized three days post treatment. The structure of the decellularized artery remained intact in comparison with the control. As can be observed in Figure 4.1, the endothelial layer has not yet recovered three days post-treatment.

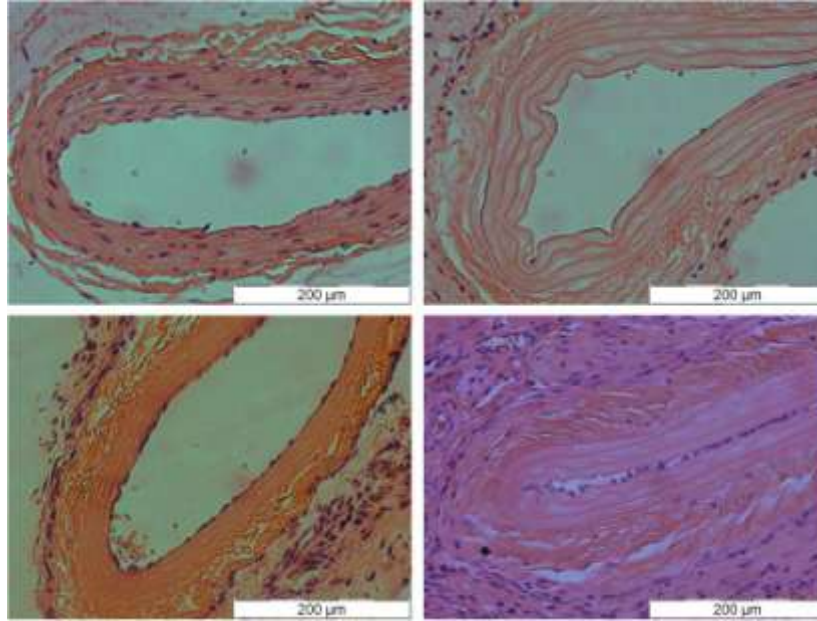


Figure 4.1. Effects of NTIRE for 1 Hz treatment. H&E staining of cell nuclei (dark purple) shows that at three days the NTIRE-treated artery (top right) is largely decellularized when compared to the control artery (top left). At five days the NTIRE-treated artery (bottom left) is decellularized, and repopulation of the endothelial layer can be seen. At seven days, the NTIRE-treated artery (bottom right panel, shown embedded in surrounding tissue) is still almost completely decellularized when compared to the control artery. Note that the endothelial cells for the treated artery at seven days are similar in number to those of the control.

After five days, histological analysis shows that the vascular smooth muscle cells are almost completely ablated when treated with the electric pulse (Fig. 4.1). Also, new cells are evident along the endothelial layer of the NTIRE-treated artery. As seen in Figure 4.1 for the 7-day group, it is evident that the artery remains mostly decellularized when treated with NTIRE. The endothelial cells provide an even coating along the inside of the decellularized artery and are similar in number to those of the non-treated control arteries.

Similar results are seen for an artery treated with a 4 Hz frequency. As can be seen in Figure 4.2, at three days after treatment the artery is almost completely decellularized and lacks an endothelial layer. By seven days, however, cells have begun to repopulate the artery and an endothelial layer is seen with a density that is close to that of the control.

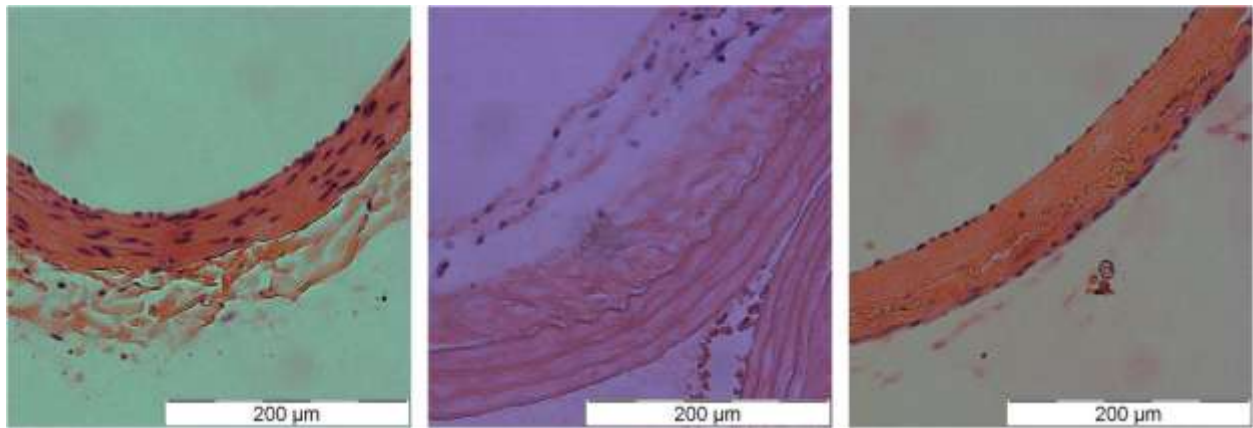


Figure 4.2. Effects of NTIRE for 4 Hz treatment. H&E staining shows the results for an artery three days after NTIRE-treatment (middle panel) and at seven days post treatment (right) as compared to the non-treated control (left). At three days, the artery is almost completely decellularized. Seven days after treatment, though still mostly decellularized, cells have begun to repopulate the artery, especially along the endothelial layer.

Further analysis focused on the 1 Hz frequency groups. At seven days it was evident that not only was the artery decellularized with cells lining the lumen, but the DNA had also been removed as illustrated in Figure 4.3.

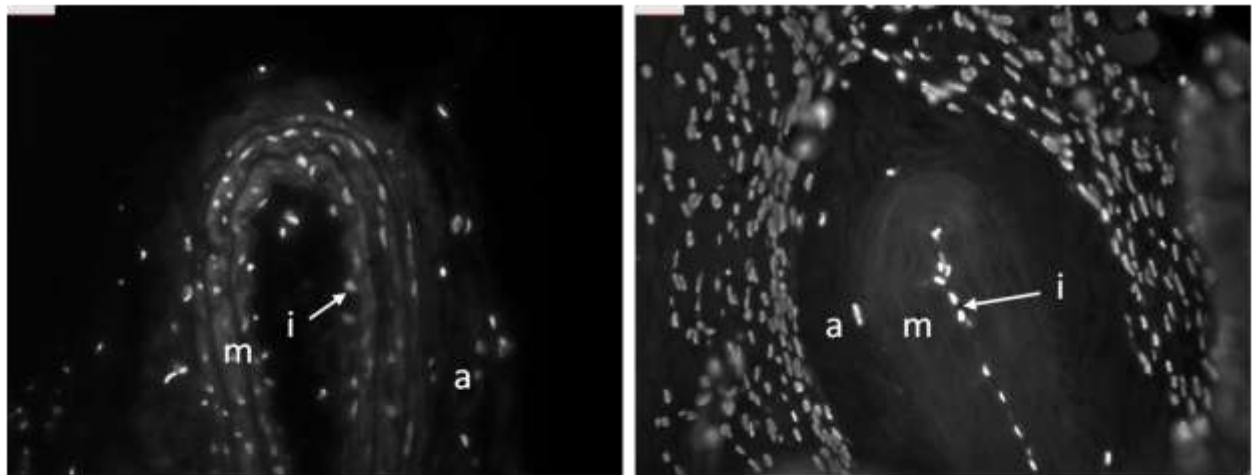


Figure 4.3. DAPI staining. Though DNA (shown by the bright spots) is seen throughout the medial (m) and adventitial (a) layers of the control artery (left panel), the medial and adventitial layers of the artery 7 days after NTIRE-treatment (right panel) are almost completely void of DNA. The NTIRE-treated artery shown here is embedded in tissue, and DAPI shows DNA throughout the tissue surrounding the artery as well as along the artery intima layer (i). The white scale bar on the top left corner of each image represents 25 µm.

Additional evidence of decellularization is demonstrated by immunohistochemical staining for α -smooth muscle actin (α -SMA). At three days, there is a decrease in α -SMA, and by seven days there is no α -SMA present, indicating a lack of vascular smooth muscle cells (VSMC) seven days after NTIRE-treatment as shown in Figure 4.4.

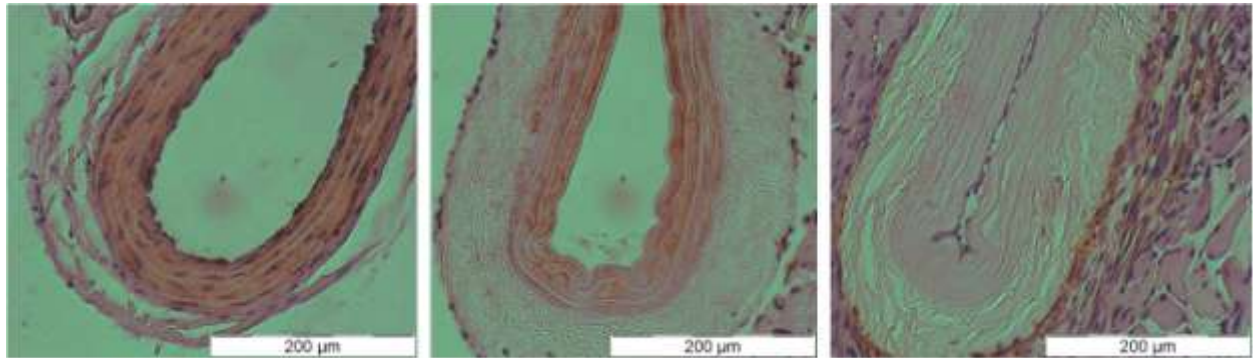


Figure 4.4. Smooth muscle cell removal. Arteries stained for α -SMA (red-orange) demonstrates a decrease in VSMC at three days (middle) and a lack of VSMC in the acellularized construct at seven days (right) after NTIRE-treatment as compared to the control (left).

In order to determine the type of cells that had begun to repopulate the lumen surface of the 7-day group, Factor VIII staining was used to identify endothelial cells, as shown in Figure 4.5.

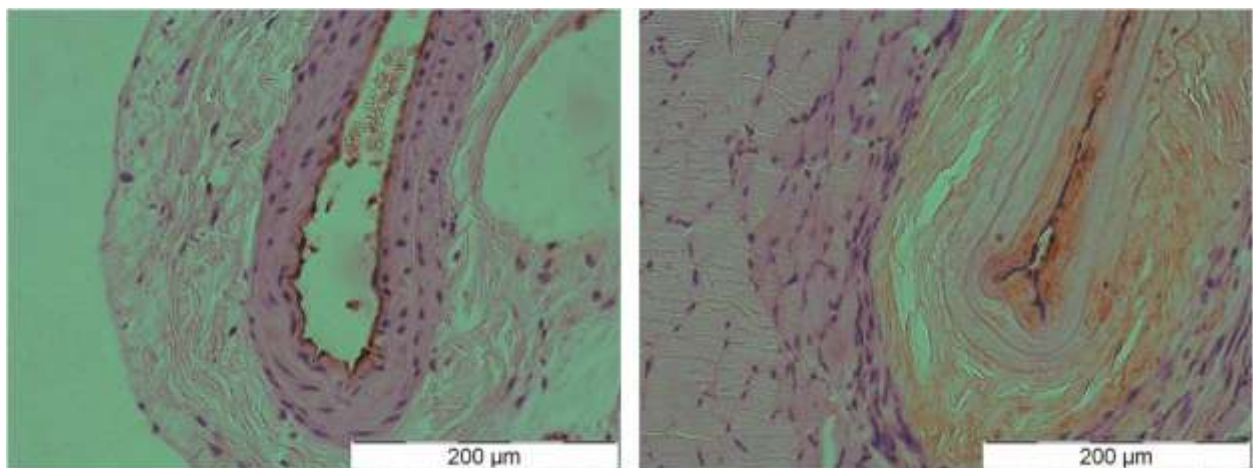


Figure 4.5. Staining for endothelial cells. Staining for Factor VIII related antigen (shown in brown) was used to identify the cells lining the lumen as endothelial cells for both the control (left panel) and the treated artery at seven days after applying NTIRE (right panel).

Histological analysis of both the 3-day and the 5-day groups revealed sections along the artery's length where the tunica media transformed from being completely populated by VSMC to being fully decellularized. These delineated sections are highlighted in Figure 4.6.

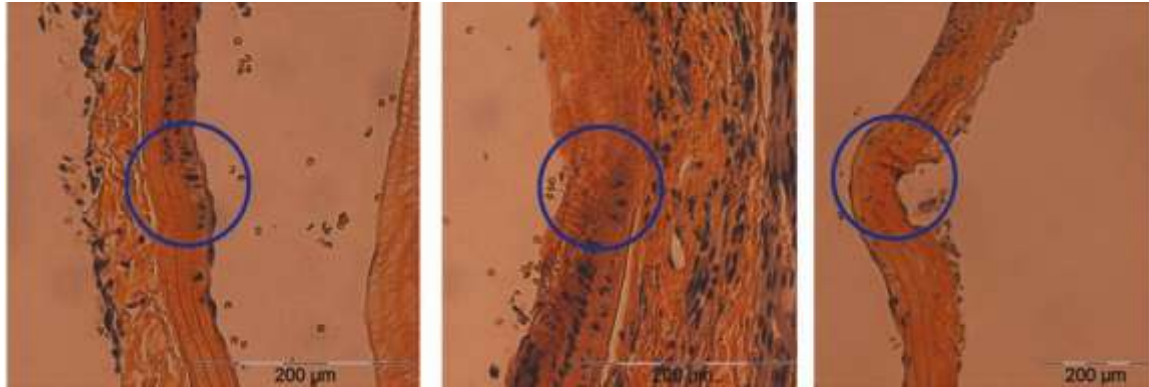


Figure 4.6. Ablation zone boundary. Marked margination between VSMC-populated and depopulated regions are highlighted in three different examples.

EVG staining at both three and seven days post treatment showed evidence of intact elastin fibers and preserved vessel wall (Fig. 4.7). Further staining with Movat's pentachrome demonstrated preservation of the extracellular matrix, especially elastin as well as collagen and proteoglycans (Fig. 4.8).

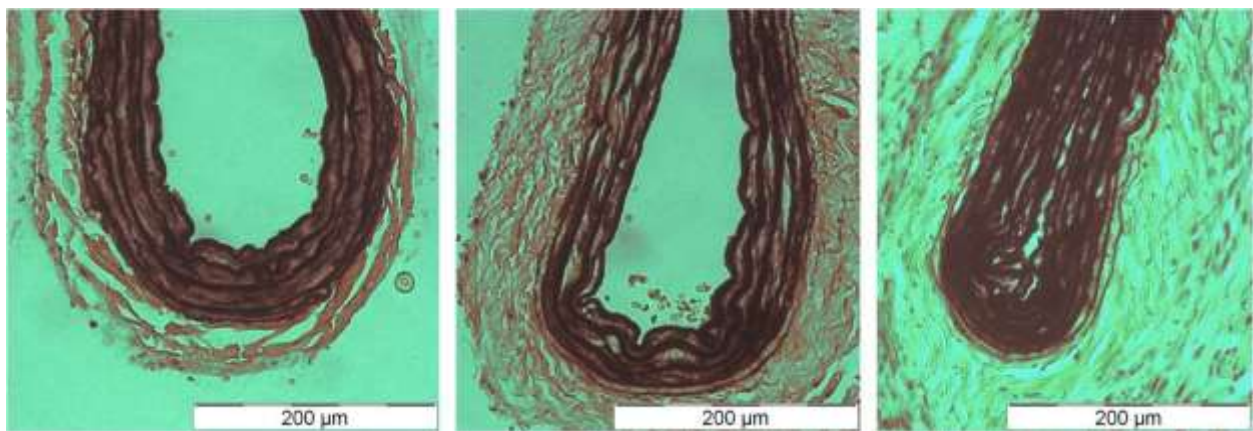


Figure 4.7. EVG staining. From the 1 Hz pulse group, EVG staining shows undamaged elastin fibers for the NTIRE-treated artery at three days post treatment (middle) and at seven days post treatment (right) when compared to the control (left).

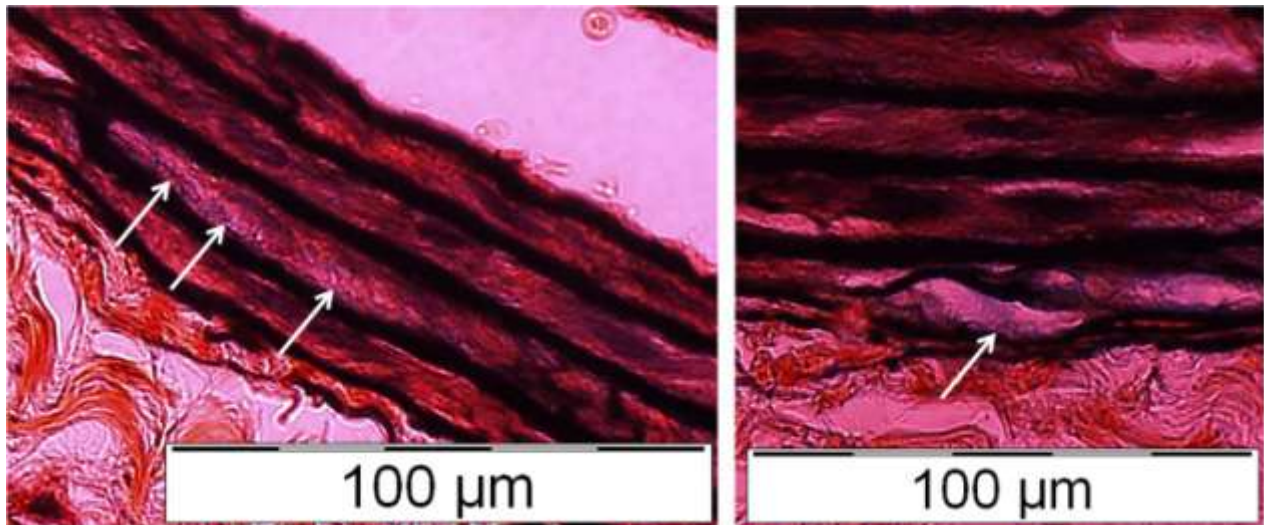


Figure 4.8. Further ECM analysis. Movat's pentachrome stain for an artery 3 days after NTIRE-treatment shows undamaged elastin fibers (black) as well as collagen and reticulum fibers (orange) and proteoglycans (blue and highlighted by arrows).

Masson's trichrome stain indicates that cell nuclei and vascular smooth muscle fibers are no longer present after NTIRE treatment. The presence of collagen fibers can also be seen, as shown in Figure 4.9.

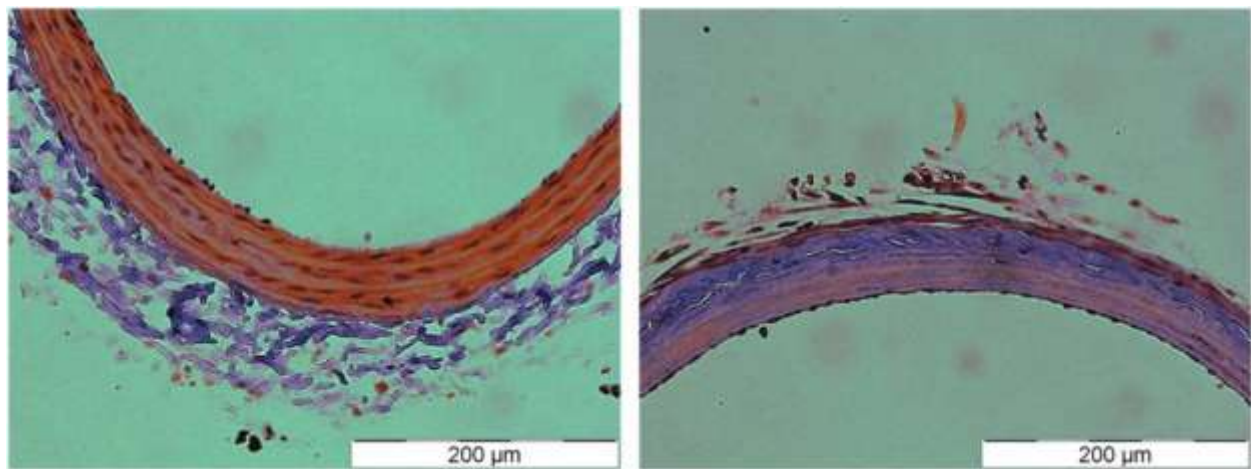


Figure 4.9. Masson's trichrome stain. Masson's trichrome stain demonstrates the absence of cell nuclei (stained dark brown) and vascular smooth muscle fibers (red) seven days after NTIRE treatment (right) as compared to the control (left). Here, it can also be seen that an abundance of collagen fibers remain after the treatment method (stained blue).

4.3.2 Rabbit Iliac Artery Using Endovascular Electrodes

Histological analysis of the rabbit iliac artery treated with the endovascular electrode device indicates that the artery becomes greatly decellularized, especially throughout the medial layer as shown in Figure 4.10. As can be seen in the figure and similar to that seen for the rat carotid artery, the endothelial layer has begun to regenerate by seven days after treatment.

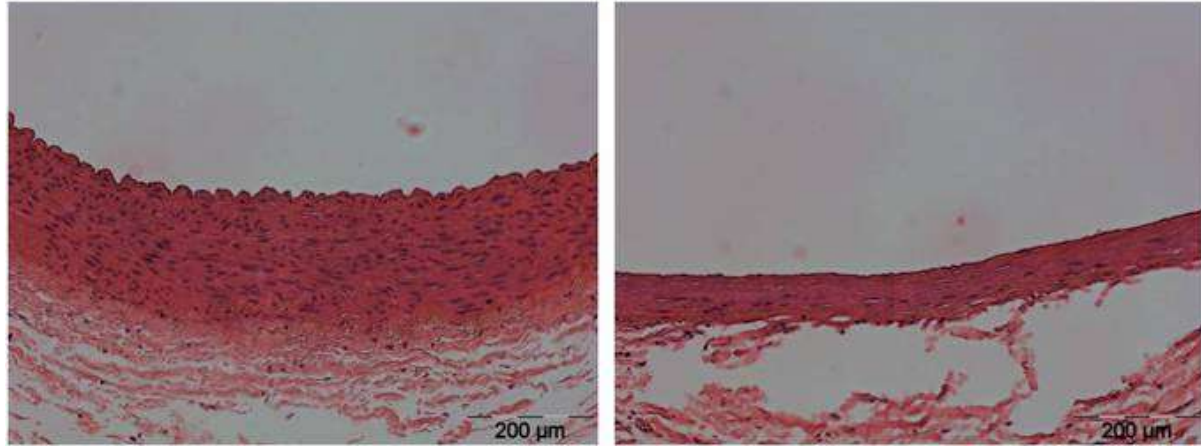


Figure 4.10. H&E staining for the rabbit iliac artery. H&E staining shows that the NTIRE-treatment of the rabbit iliac artery using the endovascular device resulted in complete absence of VSMC at one week after treatment (right) as compared to the control (left).

Further analysis with Masson's trichrome stain was used to demonstrate the loss of cell nuclei and vascular smooth muscle fibers in the treated arteries as well as the presence of collagen fibers after treatment (Fig. 4.11).

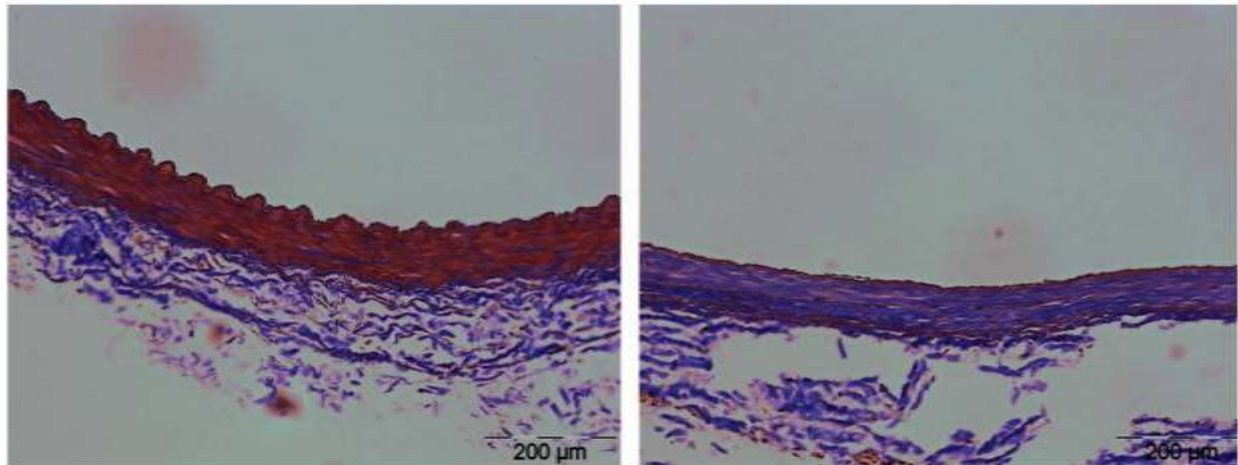


Figure 4.11. Masson's trichrome stain for the rabbit iliac artery. Masson's trichrome stain indicates an absence of cell nuclei (stained dark brown) and VSMC fibers (red) seven days after NTIRE treatment using the endovascular device (right) as compared to the control (left). Collagen fibers are stained blue, and are present both before and after treatment.

In a manner similar to that shown for the rat carotid artery, EVG staining showed intact elastin fibers as well as preservation of the vessel wall after NTIRE-treatment, as seen in Figure 4.12.

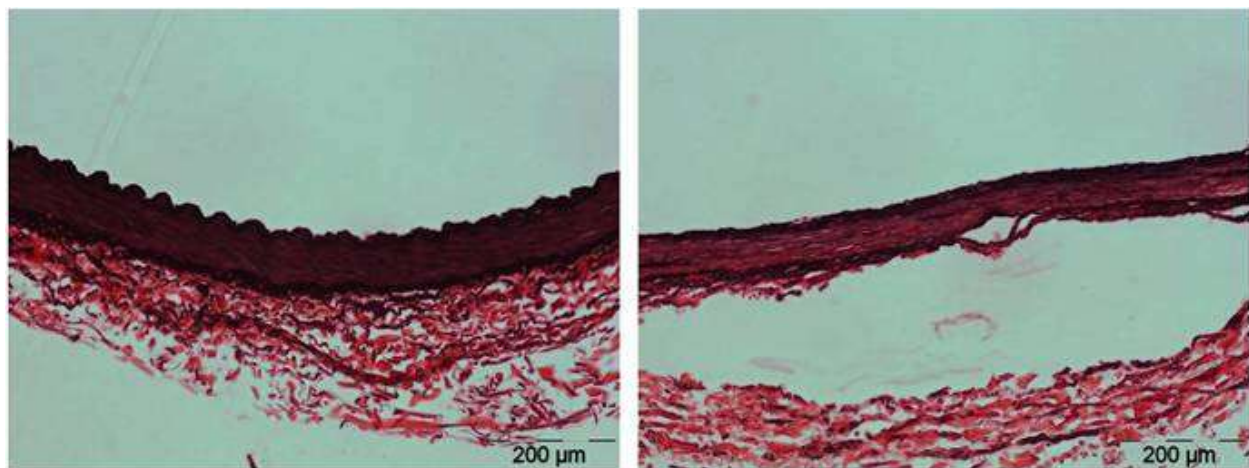


Figure 4.12. EVG staining for the rabbit iliac artery. EVG staining shows that elastin fibers remain undamaged at seven days after NTIRE-treatment (right) when compared to the control (left).

4.4 DISCUSSION

This chapter examines how NTIRE affects the artery over time and how the artery responds and reacts to the treatment. The results presented here shows that not only are the cells throughout the artery ablated by the electroporation protocol, but that the cellular material is removed from the artery naturally. This indicates that between three and five days the artery is most likely to reach its peak level of decellularization, as illustrated by H&E staining. DAPI staining further illustrates the loss of cellular material after electroporation, and, additionally, the removal of VSMC cells are shown by α -smooth muscle actin staining. At seven days after treatment, cells can be seen repopulating the lumen, and Factor VIII staining demonstrates that these are endothelial cells. In addition, staining indicates that the extracellular matrix is still intact with key components after NTIRE, and it is likely that this contributes to the artery's ability to rebuild its endothelial layer and become recellularized within a week for treatment.

4.4.1 Artery Recovery after NTIRE for Cancer Treatment

These results show that, although the cells are ablated throughout the artery after NTIRE, the artery's extracellular matrix does not appear affected by the procedure. Thus, by maintaining this structure, the artery is able to continue functioning *in vivo* as supported by previous experimental observations [Lee *et al*, 2007; Onik *et al*, 2007]. It is probable that an intact extracellular matrix, complete with important molecules such as proteoglycans (essential in regulating the movement of molecules through the matrix in addition to affecting the activity and stability of proteins and signaling molecules) is what encourages the endothelial layer to become fully recellularized within one week of treatment. Results shown here indicate that important ECM components such as collagen, elastin, and proteoglycans are retained after NTIRE as demonstrated by Movat's pentachrome stain (Fig. 4.8). After NTIRE treatment by both plate electrode and endovascular device treatment methods, there remains an abundance of collagen fibers (Figs. 4.9 and 4.11) and EVG staining indicates that the elastin fibers and vessel wall are undamaged and similar to the control after seven days (Figs. 4.7 and 4.12). These results are expected, since NTIRE selectively disrupts the cell membrane's lipid bilayer. Thermal damage is eliminated by controlling the electrical parameters and minimizing Joule heating as demonstrated in previous work [Davalos *et al*, 2005; Maor *et al*, 2008]. Mathematical modeling of the effects of the electrical parameters used in this study on the temperature of the tissue (see Chapters 2 and 3) indicate that although the maximum temperature experienced by the artery may exceed the thermal damage threshold of 315.15 K [Tropea and Lee, 1992; Dickson and Calderwood, 1980], this only occurs for a very short amount of time, and thus thermal damage is minimized. Thermal damage values obtained from utilizing the Arrhenius equation (Eq. 7) were orders of magnitude less than 1, indicating that any damage to the tissue due to Joule heating is less than 2% as a conservative damage estimate, and this lack of thermal damage is demonstrated by the intact ECM.

Perhaps the most important result of this study is the evidence that the artery has retained its function to support cell migration and growth and that the endothelial cells began to repopulate after five days and were completely regenerated after seven for the plate electrode technique (Fig. 4.4). Figure 4.10 indicates that this is also the case when the rabbit iliac artery was treated with NTIRE in a minimally invasive manner, resulting in medial and adventitia

layers that are almost completely devoid of cells, though a sparse layer of cells is evident, lining the lumen. This endothelial re-growth is very important and indicates that the arteries retain their function after NTIRE-treatment and that problems such as thrombogenicity may be avoided, an essential aspect for complete recovery in the region in which tissue ablation was applied. As illustrated in the histological analysis, both the clamp electrode device and the endovascular device result in an ECM that is able to support such cell growth within a week after treatment.

These results indicate that NTIRE can be used for tumor ablation even in the case in which important arteries are embedded within the tumor. Although cellular destruction of the artery may occur, the artery is able to maintain its important structural and extracellular components after treatment, and this enables the artery to redevelop a full endothelial layer within one week. These results show that collateral damage to the artery is minimal, and signs for recovery are evident. In addition, it is shown here that similar results are obtained by applying NTIRE to the artery using two different methods: the plate electrodes and the endovascular device. Though the endovascular device's minimally invasive application makes it more clinically relevant in some cases, the plate electrodes method is a much more practical technique for the laboratory setting, and here it can be seen that the arterial tissue responds in the same manner to both methods.

4.4.2 Applications for Tissue Engineering

In addition to illustrating the safety and ability of NTIRE to minimize collateral damage to arteries during cancer treatment, this chapter demonstrates the potential use of NTIRE and the body's host response to derive a functional decellularized tissue scaffold. Two different methods for applying NTIRE to the artery were analyzed and experimentally tested, illustrating the versatility of NTIRE as a tool for tissue engineering. Here it is shown that a decellularized artery can be developed *in vivo*, both by using an electrode clamp on the outside of the artery and by minimally invasive techniques, applying the electrical pulse from the inside of the artery using an endovascular electrode device. By applying irreversible electroporation to the artery *in vivo* and controlling the electric parameters such that thermal damage is avoided (Chapters 2 and 3), this study indicates the potential to ablate the cells within the artery wall without altering the gross structure of the ECM. This work shows that there is a period in which the artery becomes decellularized before new cells begin to grow back. This is when the decellularized tissue could potentially be harvested and implanted in the recipient. Here, the promise of such a method is shown, with the potential use of developing a decellularized artery *in vivo* which could be extracted and put to use as a potential graft for revascularization surgeries.

The results presented here indicate that between three and five days the artery is most likely to reach its peak level of decellularization, as illustrated by H&E staining, DAPI staining, and the loss of α -smooth muscle actin throughout the medial layer. For harvesting, it is important to determine a time after the ablated cells have been removed from the artery wall but before the endothelial layer begins to repopulate. The results shown here demonstrate that harvesting should be done between the third and fifth day post NTIRE treatment and that the endothelial layer begins to regenerate at seven days as indicated in Figures 4.1, 4.2, and 4.10.

Previous work in the field of tissue scaffolding has developed other potential techniques to decellularize arteries, blood vessels, and other tissues [Huynh *et al*, 1999; Clarke *et al*, 2001; Conconi *et al*, 2006; Flynn *et al*, 2006]. Most of these methods, however, require the use of chemicals and enzymes that may cause harm to the ECM, remove signaling proteins, or leave behind toxins that could reduce cell growth and lead to graft failure [Gilbert *et al*, 2006]. The results shown here illustrate that the ECM structure is preserved along with important components for promoting cell growth. The ability of NTIRE to selectively target the cell membranes while preserving the extracellular matrix is what gives it this unique ability to produce a decellularized tissue construct *in vivo*.

Evidence of the ECM retaining its structure and ability to support cell migration and growth is very important in regard to developing a decellularized tissue scaffold. Observations of endothelial cells repopulating the lumen within seven days after treatment for both the plate electrode and the endovascular electrode treatment method are important in demonstrating the potential of this decellularization technique. This indicates that the scaffold is intact and is able to encourage cell growth and that problems such as thrombogenicity experienced by many other tissue engineered grafts may be avoided.

Another potential advantage of the NTIRE-derived scaffold method is its overall simplicity and relative speed. These results show that an artery can be decellularized within less than a week using a very simple and inexpensive procedure. NTIRE is also a very predictable and controllable technology. The ablation zone is well defined as depicted in Fig. 4.6, demonstrating the clear margination between treated and untreated sections of the artery. This is consistent with previous work [Lavee *et al*, 2007; Maor *et al* 2008; Rubinsky, 2007], and indicates that NTIRE can be used to decellularize an artery without causing damage beyond the ablation zone to the surrounding tissue. Though this work has focused on decellularizing the artery, we foresee that it could be scaled up to larger, more complex tissue geometries such as the heart, using electric probes and perhaps multiple applications of NTIRE.

This study demonstrates the ability to obtain a decellularized artery for use as a tissue graft using two different application techniques: the clamp electrode applied to the outside of the artery and the endovascular electrode device applied minimally invasively. It is shown that within one week after treatment, the artery becomes decellularized. New endothelial cell growth is seen along the lumen layer, demonstrating the ECM can still support cell growth. This study illustrates that, with the support of mathematical modeling (Chapters 2 and 3), NTIRE can be used to decellularize arteries and perhaps other tissue types that differ in location, size, and species. NTIRE is a simple, controllable, and versatile cell ablation method that shows great promise in obtaining decellularized tissue constructs for use as tissue grafts. Future work would include scaling up the electroporation procedure to larger animal and human arterial grafts. This method assumes a reliance on the immune response or some other cellular or enzymatic mechanism to remove the dead cells. This response could potentially cause remodeling to the ECM, and thus it will be important to further assess the effects of the host response on the scaffold and to gain a deeper understanding the mechanisms involved on the cellular level.

4.5 CONCLUSIONS

Here it is shown that when NTIRE is applied to the artery by either plate electrodes or in a minimally invasive manner with an endovascular device, the cells within the artery become depleted within three to five days. In addition, a full endothelial layer is observed within one week. These results have significant implications both for investigating how the artery near or embedded within a tumor is able to recover after NTIRE treatment as well as for examining the potential use of NTIRE in developing a decellularized tissue scaffold. The ability for the extracellular matrix to retain important features and support new cell growth is important for both of these applications. For the field of cancer ablation, this indicates that damage to arteries within the ablation zone is minimal and that the artery is able to encourage new cell growth for recovery. In addition, it is demonstrated here that NTIRE and the body's host response have the potential to decellularize an artery for future tissue scaffold use, and it has been shown here that the artery ECM is able to facilitate endothelial cell growth 7-days post treatment. Although substantial further investigation is necessary to fully develop and use this technology, NTIRE is a promising method for tissue engineering which, as a first application, may prove useful in deriving a construct for use in revascularization surgeries, meeting a need that autologous and synthetic grafts cannot fully reach and resulting in a successful implantation without further complications.

CHAPTER 5: THEORETICAL ANALYSIS OF NTIRE APPLIED TO THE SMALL INTESTINE

5.1 INTRODUCTION

The previous chapters focused on the ability of the artery to recover after NTIRE, examining both applications for cancer treatment as well as the potential for developing a decellularized arterial tissue scaffold. Here, in conjunction with the theoretical and experimental results obtained for the artery, another critical tissue is examined for cancer ablation applications: the small intestine.

As previously mentioned, NTIRE has been demonstrated as a successful method for treating both benign and malignant tumors in large and small animal models as well as in clinical trial [Al-Sakere *et al*, 2007; Neal *et al*, 2010; Edd *et al*, 2006; Onik *et al*, 2007; Ellis *et al*, 2011; Onik and Rubinsky, 2010]. These studies have proven that NTIRE can be advantageous over other local ablation methods such as radiofrequency and cryoablation. For example, while radiofrequency and cryosurgery both will result in destruction of nearby blood vessels and other important structures due to the thermal nature of their ablation modality, NTIRE has been shown to preserve these important structures. Through the potential side effects and collateral damage on adjacent, normal tissues have been investigated in great detail for radiation therapy [Ciorba and Stenson, 2007; Kountouras and Zavos, 2008; Packey and Ciorba, 2010; Famularo *et al*, 2010; Smith and DeCosse, 1986], very little has been done to investigate the effects of NTIRE on adjacent tissues.

Collateral damage to the small intestine often occurs after radiotherapy for pelvic or abdominal malignancies as well as a side effect of chemotherapy [Han *et al*, 2011; Keefe *et al*, 2000; Ciorba and Stenson, 2009], and this damage can often be bad enough to stop cancer treatment [Keefe *et al*, 2000; Packey and Ciorba, 2010]. Since the small intestine may be especially susceptible to treatment methods, it is important to examine how the small intestine responds to NTIRE in order to establish the safety of using NTIRE for treating pancreatic cancer and other abdominal malignancies.

Energy dissipation of high electric fields can cause an increase in the temperature of the tissue due to Joule heating [Chang and Nguyen, 2004]. This biothermal effect depends on the electrical parameters used. For the application of electroporation, electric fields can elevate the tissue temperature to a level in which the cells become damaged by thermal effects, or it can result in cell death by electroporation mechanisms with only a slight temperature increase that does not result in thermal damage [Lavee *et al*, 2007]. Prior to performing *in vivo* experiments on the effects of electroporation on the small intestine, it is important to choose electrical parameters that result in this non-thermal irreversible electroporation (NTIRE). Since, thus far, there have been no experiments that study the effects of electroporation on the small intestine, pre-experimental studies that predict potential electrical fields and thermal effects are important in developing a proper set of electroporation parameters. Here, it was desired to choose parameters that resulted in a high enough electric field to guarantee electroporation while avoiding parameters that may cause thermal damage to the tissue. This chapter analyzes the electrical and thermal effects that occur when applying NTIRE to the small intestine, and these

results are then used to choose electroporation parameters for *in vivo* experiments on the rat small intestine. The plate electrodes used on the small intestine are similar to those used to apply electroporation across the artery. As can be seen in Figure 5.1, these plate electrodes consist of two electrode blocks of stainless steel that are used to gently press across the small intestine.



Figure 5.1. Plate electrode used to apply NTIRE across the small intestine. The plate electrodes used for *in vivo* experiments on the rat intestine are shown. These BTX Caliper Electrodes are produced by Harvard Apparatus (Holliston, MA). When applied across the rat small intestine, the two electrodes are held apart by approximately 1 mm.

5.2 METHODS

In order to choose electrical parameters for experimental use that would not cause extensive heating and thermal damage to the tissue, a transient finite element analysis was performed, modeling the effect of Joule heating on the temperature distribution in the intestinal tissue. The results were then used to determine the accumulated thermal damage in the tissue over time and to ensure that the electrical parameters modeled would minimize thermal damage to the tissue. A commercial finite element package (Comsol Multiphysics 3.5a) was used to develop the model and plan the electrical treatment parameters. The small intestine and plate electrodes were modeled two-dimensionally as illustrated in Figure 5.2. The small intestine's dimensions were based on experimental observations as well as data from literature [Dou *et al*, 2002]. The plate electrodes and small intestine are held close to the body during the procedure, and thus the system was modeled as being surrounded by air at an elevated temperature of 37°C.

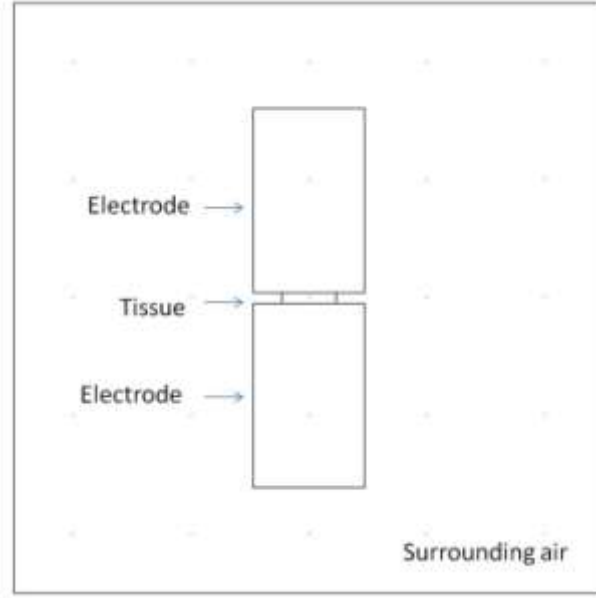


Figure 5.2. Tissue and electrode model geometry. The small intestine was modeled two-dimensionally as a 4.63 mm x 1 mm rectangle pressed between two stainless steel electrodes (each of 9.4 mm x 15.6 mm) and held within a 5 cm x 5 cm air space.

The thermal and electrical properties of the small intestine were assumed to be both isotropic and homogeneous in cross-section. This model followed the analysis described by Phillips *et al* [Phillips *et al*, 2011] and detailed in Chapter 2 for treatment of the rat carotid artery by a plate electrode device. Briefly, the Laplace equation ($\sigma \nabla^2 \phi = 0$) was solved in order to determine the heat generation per unit volume due to Joule heating (q_{JH}):

$$q_{JH} = \sigma |\nabla \phi|^2 \quad (5.1)$$

where ϕ is the electric potential and σ is the electrical conductivity. The top electrode was set as having a positive potential ($\phi_1 = V_o$) and the bottom electrode was set as ground ($\phi_2 = 0$), where V_o is the potential difference applied across the electrodes. The boundaries between the electrodes and air and between the small intestine and air were set as electrically insulating. The resulting heat generation term (q_{JH}) was then used as the heat source term in the heat conduction equation in order to solve for the temperature distribution in the tissue.

$$\rho C \frac{\partial T}{\partial t} - \nabla \cdot (k \nabla T) = q_{JH} \quad (5.2)$$

Here ρ is the material density, C is the heat capacity, and k is the thermal conductivity. The entire system was initially held at the physiological temperature of 37°C, and the edges of the air space were held at 37°C, providing a conservative overestimate of the temperature.

In this model, the full procedure utilized N square dc pulses of t_1 μ s each and a pulse frequency of f at a given electrical potential. Electrical and thermal properties used for the tissue and electrodes are given in Table 5.1.

Table 5.1 Electrical and Thermal Properties Used in Model for Tissue and Stainless Steel Electrodes.

			Tissue	Electrodes
Electrical conductivity	σ	S/m	0.6 [Gabriel <i>et al</i> , 1996]	4.032×10^6
Heat capacity	C	J/kg-K	3,750 [Davalos <i>et al</i> , 2003]	475
Density	ρ	kg/m ³	1,000 [Davalos <i>et al</i> , 2003]	7850
Thermal conductivity	k	W/m-K	0.5 [Davalos <i>et al</i> , 2003]	44.5

The temperature increases during each pulse due to the resistive heating and is dissipated due to conduction to the electrodes and to the surrounding air. In order to solve for the temperature distribution over the entire procedure and thus find a measure of the resulting thermal damage to the tissue, Matlab R2009b was used to run Comsol Multiphysics 3.5a. The coupled electric field and heat conduction equations were solved at the end of each pulse and after each interval between pulses. The maximum tissue temperature at each time step was stored as well as once every second for three minutes after the last pulse. The maximum temperature values were then used to calculate the thermal damage to the tissue using the Henriques and Moritz thermal damage integral [Diller and Pearce]:

$$\Omega(t) = \int A \exp\left(-\frac{\Delta E}{RT}\right) dt \quad (3)$$

where t is the time in seconds, R is the ideal gas constant, A is the measurement of molecular collision frequency, and ΔE is the activation energy for the molecules to denature. A and ΔE are typically determined experimentally. Since no values could be found in the literature specifically for small intestinal tissue, values determined for arterial tissue molecules [Agah *et al*, 1994; Wright, 2003] were used here in order to gain a rough estimate of the potential thermal damage, giving $A = 1.552 \times 10^{67} \text{ s}^{-1}$ and $\Delta E = 4.3 \times 10^5 \text{ J/mol}$. Ω is the damage parameter and can be expressed as the logarithm of the ratio of the undamaged molecules before the procedure to the undamaged molecules at a given time. Thus, calculating Ω can give an estimate of the percentage of thermal damage that occurs throughout the procedure.

In order to determine which electrical parameters would be work best experimentally to produce complete electroporation ablation throughout the tissue while avoiding thermal damage, a range of electrical parameters were modeled. From these simulations, the resulting maximum tissue temperature and percent thermal damage were compared. The sets of electrical parameters chosen for simulation were similar to those known to cause irreversible electroporation (parameters numbers 1-5), and additional parameters were used to examine the effect of lowering the number of pulses applied (parameters numbers 6 and 7). These parameters are given in Table 5.2.

Table 5.2. A sample of electrical parameters modeled.

Parameter Set Number	Number of Pulses	Pulse Length [μ s]	Frequency [Hz]	Electric Potential [V]
1	50	100	4	200
2	50	70	4	200
3	50	70	1	200
4	50	70	1	250
5	50	70	4	250
6	25	70	4	250
7	20	70	4	250

5.3 RESULTS

The maximum temperature obtained in the tissue over the entire electroporation procedure was determined from the resulting thermal distribution. Equation 3 was also applied over the entire procedure, giving the thermal damage parameter and resulting percent thermal damage at the location of maximum temperature increase. These results are given in Table 5.3, corresponding to each set of electrical parameters described in Table 5.2.

Table 5.3. Maximum temperature and damage results for electrical parameters modeled. The maximum tissue temperature obtained from the small intestine thermal distribution over the entire simulation is given, as well as the corresponding thermal damage parameter and resulting percent damage that is expected to occur.

Parameter Set Number	$T_{\max}$ [$^{\circ}$ C]	Damage	% Damage
1	40.51	0.0018	0.18
2	39.46	0.0015	0.15
3	38.21	0.0017	0.17
4	38.71	0.0020	0.20
5	40.83	0.0019	0.19
6	40.53	0.0014	0.14
7	40.45	0.0014	0.14

As can be seen by the results (Table 5.3), all sets of electrical parameters resulted in very low thermal damage, keeping any tissue damage well below 1%. From this analysis, although any parameter set would work between Numbers 1-5, parameter set Numbers 2 and 3 appear to give the best results in minimizing thermal damage. Although parameters set Number 3 results in a lower maximum temperature, it experiences a slightly higher thermal damage since the procedure is applied over a longer period of time. Though either of these cases would be

acceptable for *in vivo* studies in regard to minimizing thermal damage, parameter set Number 2 was chosen. This is because parameter set Number 2 not only minimized any thermal damage effects, but, since it incorporates a 4 Hz frequency, the time spent applying the electrical pulse during surgery will be much faster, minimizing the small intestine exposure time. Thus, based on these simulations, an electrical pulse parameter set of 50 pulses of 70 μ s each and 200 V with a 4 Hz frequency was chosen, corresponding to a resulting electric field of 2000 V/cm. These electrical parameters result in a maximum tissue temperature of 39.46 °C (corresponding to a temperature increase of 2.46°C) and a 0.15 % predicted thermal damage. The resulting electric potential distribution for these parameters is shown in Figure 5.3, and the maximum temperature distribution (taken immediately after the 50th pulse) is given in Figure 5.4.

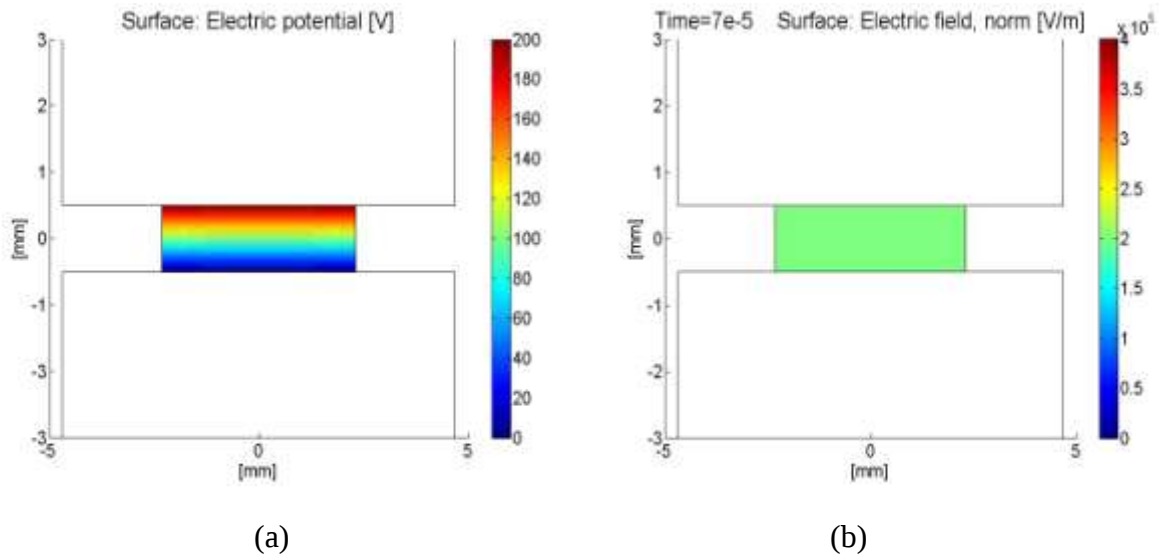


Figure 5.3. The electric potential and resulting electric field. (a) The electric potential experienced by the small intestine model for electrical parameters in parameter set Number 2 is shown, varying throughout the tissue from 200 V to 0 V. (b) The resulting electric field is uniform, with all small intestinal tissue experiencing 2000 V/cm from the applied electric parameters.

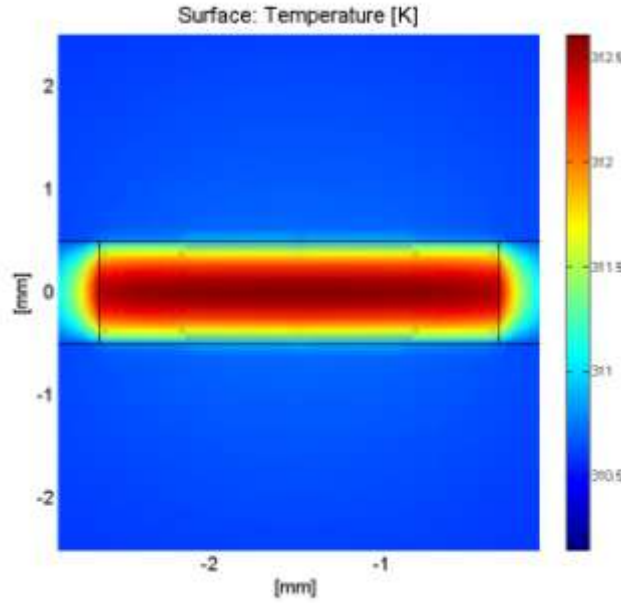


Figure 5.4. Maximum temperature distribution. The temperature distribution throughout the tissue is taken immediately after the 50th pulse.

The maximum temperature was stored at the end of each pulse and after each interval as well as once every second during the cool down period. These temperature values were used to calculate the thermal damage parameter, and are plotted in Figure 5.5.

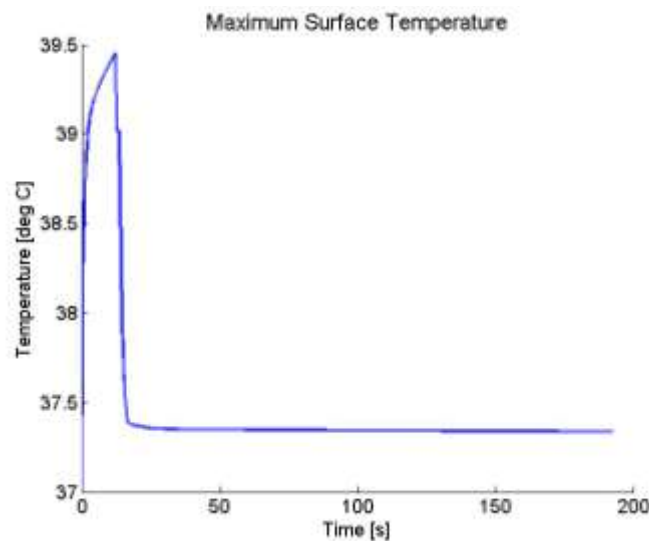


Figure 5.5. Maximum temperatures obtained over the course of the simulation. The maximum temperatures obtained at time steps throughout the simulations show a peak maximum temperature obtained after the final electric pulse followed by a rapid cool down period.

5.4 DISCUSSION AND CONCLUSIONS

Using a combined analysis of the electrical and thermal effects that would occur in the small intestinal tissue during NTIRE, different sets of electrical parameters were modeled. A sample of these parameters is given in Table 5.2. Here, finite element analysis was used to obtain the maximum temperatures that the tissue would experience both during and after the electroporation treatment. A measure for the percent of thermal damage that would occur in the tissue was then calculated using the Henriques and Moritz thermal damage integral. The tissue damage corresponding to the sets of electroporation parameters are shown in Table 5.3. It can be seen that all sets of electroporation parameters result in less than 0.2 % thermal damage to the tissue and would be safe to use. The electroporation set chosen minimized both the thermal damage to the tissue and the amount of time over which the electroporation pulses were applied. Thus, from this analysis, an electroporation protocol of 50 pulses of 200 V and 70 μ s each would be used to provide an electric field of 2000 V/cm across the small intestine with a frequency of 4 Hz. The finite element analysis used in this study predicts that these electroporation parameters will result in a maximum temperature increase of about 2.5 °C and about 0.15 % thermal damage to the tissue.

These electroporation parameters provide an electric field that is well within the range expected to produce irreversible electroporation effects on biological tissue. Thus, using these electroporation parameters *in vivo*, it is expected that complete cell ablation by electroporation will occur while sparing the extracellular matrix and important tissue proteins and structures from thermal damage. All electroporation parameter sets modeled showed predictions for very little thermal damage. This is due to the electrodes used to apply electroporation across the small intestine. As can be seen in Figure 5.5, the tissue cools down very quickly after the last pulse is applied, due to the comparatively large stainless steel electrodes which quickly conduct the heat away from the small intestinal tissue. The ability for the tissue to cool down in such a quick manner ensures that very little thermal damage occurs and explains why the predicted thermal damage is so low.

In conclusion, this model of the small intestine was utilized in order to quickly evaluate different sets of electroporation parameters, helping to choose an electroporation protocol that can cause irreversible electroporation damage to the small intestine while avoiding thermal effects. For these small intestine studies, the goal is to develop an electroporation protocol that is well above the irreversible electroporation threshold, simulating a case in which the small intestine receives a high level of electroporation, perhaps due to its proximity to a tumor that is undergoing irreversible electroporation ablation. In Chapter 7, these electroporation parameters will then be tested *in vivo* in order to assess how the small intestine responds to cell ablation by irreversible electroporation. Here, we are focused on the unique ablation method of NTIRE, and thus electroporation parameters had to be modeled prior to testing *in vivo* in order to ensure that they would result in a high electric field while minimizing resistive heating to the tissue. The set of electroporation parameters chosen in this study meet this goal. It is hypothesized that, since thermal effects are avoided, the extracellular matrix will remain undamaged after electroporation, enabling the small intestine to recover quickly. This hypothesis is investigated experimentally in Chapter 7.

CHAPTER 6: MODELING THE SMALL INTESTINE AS A HETEROGENEOUS TISSUE

6.1 MOTIVATION

The homogenous tissue model used to predict the electric and thermal fields in the small intestine (Chapter 5) contained many simplifications. Though this analysis was useful in providing a quick method in making an informed decision on the set of electrical parameters that could be used *in vivo* to cause tissue ablation to the small intestine, in many situations it may be beneficial to have a model that includes fewer simplifications, taking into account the layered structure of the small intestine. This is especially true for treatment planning. When using irreversible electroporation for cancer treatment, researchers and surgeons wish to develop a treatment plan that can ablate the cancerous tissue while sparing as much of the non-cancerous, healthy tissue as possible. Numerical modeling is important in predicting which areas of the tissue will be treated by irreversible electroporation. Should irreversible electroporation be used to treat abdominal cancers such as a tumor adjacent to the small intestine, it is important to be able to include a more detailed analysis of the tissue when developing numerical predictions for planning the treatment parameters.

Heterogeneous tissue can strongly affect how the electric field is distributed. It is expected that, in reality, non-homogeneous electric fields will occur throughout the small intestinal tissue due to the heterogeneous nature of the layered intestine. The small intestine layers are illustrated in Figure 6.1.

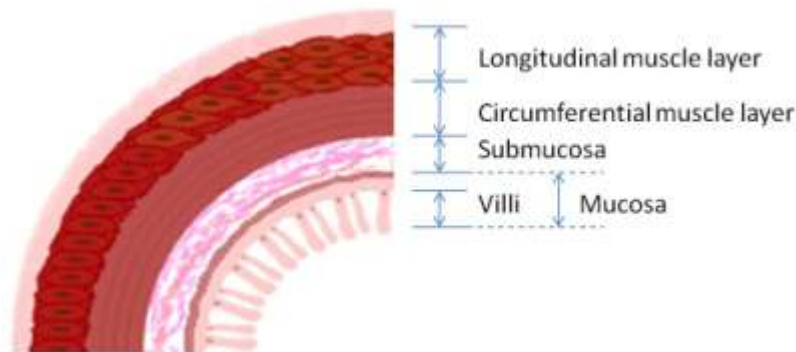


Figure 6.1. Typical layers of the small intestine. This image (not to scale) depicts the layers of the small intestine that were modeled for electric field and thermal analysis.

Here, the effect of changes in electrical conductivity from one layer to another is investigated. Since the actual electrical conductivities of the tissue could not be found in the literature, a parametric study was utilized to gain an understanding of how the electric field

distribution can potentially develop. Numerical models of heterogeneous tissues are a necessary step in developing irreversible electroporation for clinical treatment of abdominal cancers.

6.2 SMALL INTESTINE MODEL

6.2.1 Model Geometry

Dimensions used for small intestine modeling were taken from both literature and from observations of the experimental conditions. From Dou *et al.* [Dou *et al.*, 2002], the dimensions of the rat small intestinal layers was determined. These were obtained from microscopic evaluations and the ileum dimensions are given in Table 6.1.

Table 7.1. Dimensions used for Model Geometry

Tissue Layer/Measurement	Dimension
Crypt depth	0.28 mm
Villus height	0.28 mm
Mucosa	0.48 mm
Submucosa	0.04 mm
Circumferential muscle	0.05 mm
Longitudinal muscle	0.04 mm
Wall thickness / Inner circumferential ratio (no load state)	0.11 mm
Wall thickness (no load state)	0.75 mm

The inner circumferential length (ICL) was calculated from the wall thickness/ICL ratio. During the experimental procedure, the small intestine is pressed gently between two plate electrodes. Thus, the centerline where the small intestine is pressed against itself was taken as half the inner circumferential length, as illustrated in Figure 6.2.

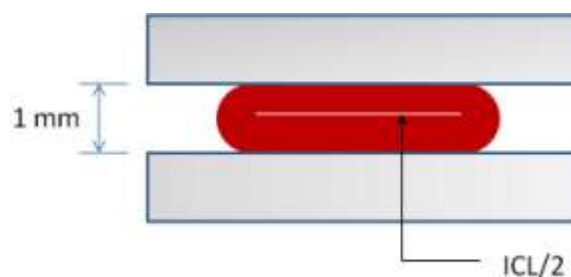


Figure 6.2. Schematic of the small intestine pressed between two electrodes. The midline length was taken as half of the inner circumferential length (ICL).

Also seen in Figure 6.2, the electrodes were held apart by approximately 1 mm, gently pressing down on the small intestine. This pressure compressed the villi layer, resulting in a modified, more compact, layer (Fig. 6.3).

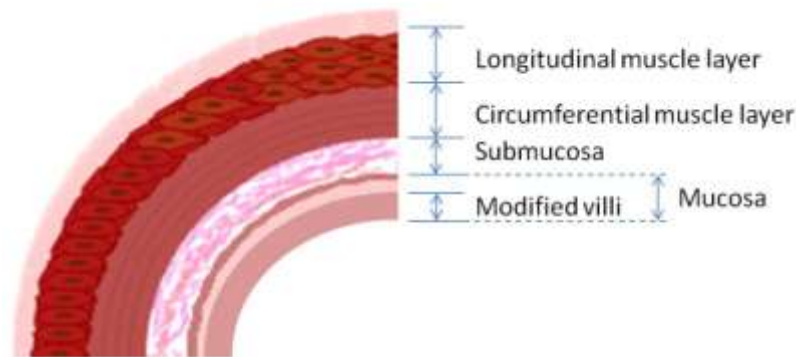


Figure 6.3. The modified villi layer. Here, the small intestine villi layer is modified to account for the villi being more compact when pressed between the two electrodes during electroporation.

Using the small intestine dimensions given in Table 6.1, the modified villi layer was calculated to be 0.17 mm thick while pressed between the two electrodes. By incorporating these assumptions along with the dimensional data given in Table 1, the small intestine geometry was built in Comsol (Comsol Multiphysics 3.5a), as shown in Figure 6.4. Here, only half of the small intestine is modeled, taking advantage of the small intestine symmetry.

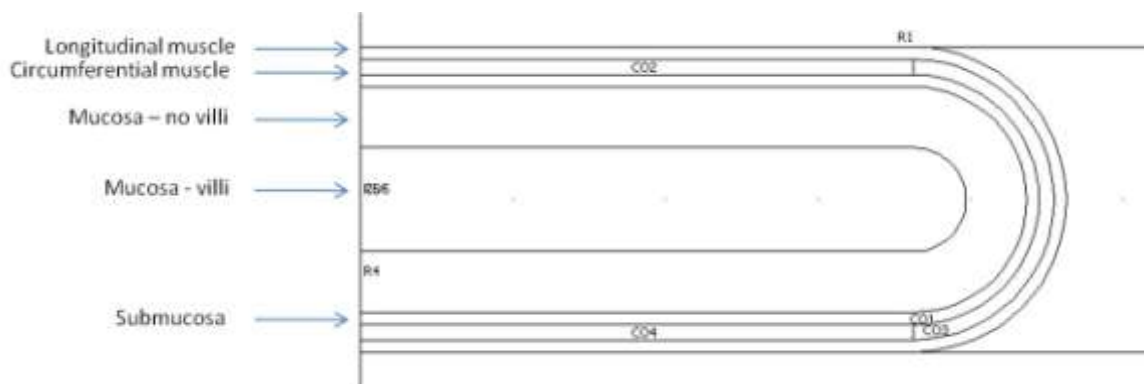


Figure 6.4. Comsol model of the small intestine geometry. Here, only the right side of the small intestine is modeled, taking advantage of the small intestine symmetry. The small intestine is pressed between two parallel electrodes, and the different intestinal layers are shown to scale.

The small intestine and electrodes are shown in full in Figure 6.5. The small intestine/electrode setup is symmetric across the vertical plane, and only the right side was modeled for simulation.

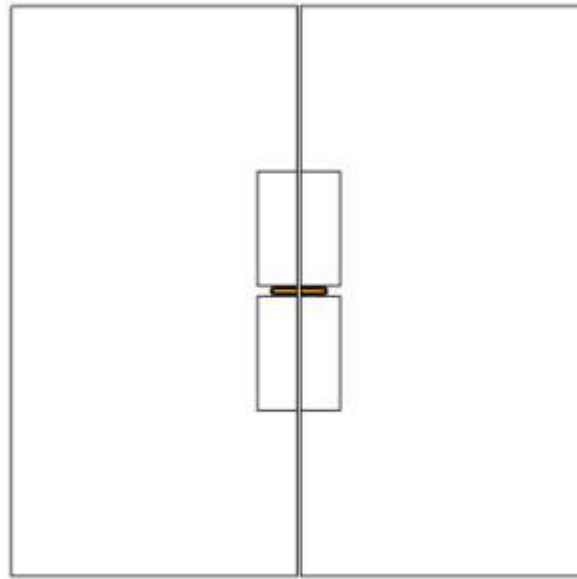


Figure 6.5. The small intestine and plate electrode model geometry. Since the small intestine and electrodes are symmetric across the midline plane, only the right side was modeled for analysis.

6.2.1 Thermal and Electrical Properties

The thermal properties for the tissue were taken from literature [Davalos *et al*, 2003]. For this analysis, all layers of the small intestine were given the same thermal properties. These values are shown in Table 6.2.

Table 6.2. Tissue thermal properties used in the analysis. [Davalos *et al*, 2003]

Thermal property		
Specific heat	c_p	3750 [J/kgK]
Density	ρ_t	1000 [kg/m ³]
Thermal conductivity	k_t	0.5 [W/mK]

The electric field and resulting temperature profile depend strongly on the electrical parameters used. Since the electrical conductivity is not available in literature for the specific

layers of the small intestine, a parametric study was performed using a range of electrical conductivity values. The electrical conductivities of the mucosa and submucosa were assumed to be directionally independent. The electrical conductivity of the muscle layers, however, is anisotropic and depends on the direction of the muscle cells in relation to the applied electric field [Bhattacharya and Mahajan, 2003]. The applied electric field will run in the vertical direction between the two parallel plates, as illustrated in Figure 6.6.

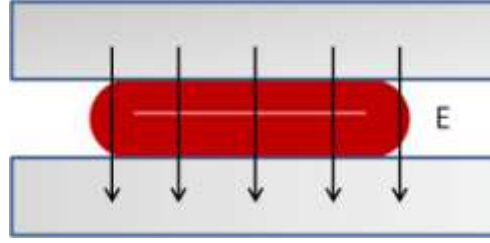


Figure 6.6. Direction of applied electric field. Here, it is shown that the applied electric field moves across the small intestine, perpendicular to the small intestine axis.

The electrical conductivity for muscle fibers arranged parallel to the electric field (σ_{II}) varies considerably from the electrical conductivity for muscle fibers oriented perpendicular to the electric field (σ_T). In the small intestine, the longitudinal muscle layer consists of muscle cells that are all oriented along the axis of the small intestine and perpendicular to the electric field. Thus, the entire longitudinal muscle layer is given an electrical conductivity of σ_T . The muscle cells of the circumferential muscle layer, on the other hand, are oriented either perpendicular to the electric field or offset from the electric field, depending on the location. In order to incorporate this dependence, a rotation matrix could be used to define the value of the resulting electrical conductivity in the x and y directions at each location along the small intestine.

$$\begin{bmatrix} \cos\theta & -\sin\theta \\ \sin\theta & \cos\theta \end{bmatrix} \begin{bmatrix} \sigma_T \\ \sigma_{II} \end{bmatrix} = \begin{bmatrix} \sigma_1 \\ \sigma_2 \end{bmatrix} \quad (6.1)$$

Here, θ is defined as the angle between the horizontal and the radius from the center of the curved section of the small intestine layer and the location along the curve, as illustrated in Figure 6.7.

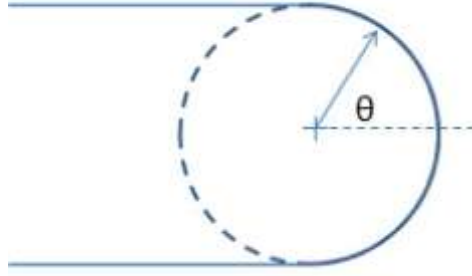


Figure 6.7. Schematic illustrating how θ was defined for the rotation matrix. Here the arrow points to the region of interest along the circumferential muscle layer curve, and θ is defined as the angle between the radius arrow and the horizontal.

This directional dependence was then be incorporated into the subdomain definitions for the circumferential muscle layer, giving

$$\sigma_{cm} = \begin{bmatrix} \sigma_2 & 0 \\ 0 & \sigma_1 \end{bmatrix} \quad (6.2)$$

Though this dependence resulted in the correct electric field distribution along the circumferential muscle layer, there were difficulties in solving the electric field distribution in Comsol due to issues with mesh size. In order to keep the mesh size such that the solution did not require a large increase in memory and time, an approximation for the muscle layer directional dependence was used:

$$\sigma_{cm} = \sqrt{(\sigma_u \cos\theta)^2 + (\sigma_T \sin\theta)^2} \quad (6.3)$$

From inspection, this resulted in the same trend in electrical conductivity distribution throughout the circumferential muscle layer, while providing a solution in Comsol that did not encounter the same meshing issues.

6.2.3 Electric Field Solution

In order to solve for the resulting electric field after applying full electric parameters to the small intestine tissue, a single electroporation pulse was first modeled using the Laplace equation:

$$\nabla \cdot (\sigma \nabla \phi) = 0 \quad (6.4)$$

Here ϕ is the electric potential and σ is the electrical conductivity at a specific location. The electrodes were represented by a fixed (Dirichlet) boundary condition. The top electrode was set as having a positive potential and the bottom electrode was set to zero:

$$\phi_1 = V_o \quad (6.5)$$

$$\phi_2 = 0 \quad (6.6)$$

where V_o is the potential difference applied across the electrodes during the electroporation pulse. In order to match the electroporation parameters chosen in Chapter 5, the electric potential was set at 200 V, corresponding to an electric field of 2000 V/cm. The boundaries between the small intestine and the air as well as between the electrodes and air were set as electrically insulating.

The Laplace equation was used to solve for the electric field distribution. In addition, Equation 6.4 can be solved for the heat generation per unit volume (q_{JH}):

$$q_{JH} = \sigma |\nabla \phi|^2 \quad (6.7)$$

6.2.4 Thermal Solution

During the surgical procedure, a segment of the ileum is pulled out of the abdominal cavity through an abdominal incision and held away from the body. The plate electrodes are then used to gently press across the small intestine to apply the electrical pulses. Thus, the artery and electrodes were modeled as being surrounded by air at 20 °C and experiencing an assumed natural convection with a convection coefficient of 10 W/m²K. Since the small intestine segment is out of the body and it has been observed by others that blood flow is temporarily stopped due to vasoconstriction during electroporation [Gehl *et al*, 2002], heat loss due to blood flow and metabolism effects were ignored. (This will actually result in a conservative, over estimate of the thermal effects, since it neglects blood flow heat loss even after the electric pulse has been removed.) Thus, the general heat conduction equation was used:

$$\rho C \frac{\partial T}{\partial t} - \nabla \cdot (k \nabla T) = q_{JH} \quad (6.8)$$

where ρ is the material density, C is the heat capacity, and k is the thermal conductivity. The heat generation due to Joule heating from the electrical pulses, q_{JH} , is determined from the Laplace equation and is given in Equation 6.7. In order to solve for the resulting temperature distribution, the small intestine and electrodes were initially held at the physiological body temperature of $T_o = 37^\circ\text{C}$. The internal boundaries between the small intestinal layers and electrodes were defined as thermally continuous, and the boundaries at the mid-plane were set as thermally insulating due to symmetry. The external boundaries of the small intestine and electrodes were defined as experiencing surface convection:

$$\mathbf{n} \cdot (k \nabla T) = h_{conv} (T_\infty - T) \quad (6.9)$$

Here $\mathbf{n}$ is the direction normal to the surface, h_{conv} is the convection coefficient due to natural convection, and T_∞ is the temperature of the surrounding air.

6.2.5 Determining Electrical Field and Thermal Damage for Pulse Sequence

The full procedure utilized N number of square dc pulses of length t_l and a pulse frequency rate of f . In order to model the changes in electric field and temperature distribution over the course of the pulse sequence, the small intestine model described above was modeled using Comsol Multiphysics 3.5a. This Comsol solution was run in Matlab for the multiple pulse protocol. A finite element mesh was incorporated that utilized triangular elements, and the mesh size was varied in order to validate the accuracy of the solution. The coupled electric field and heat transfer equations were solved at each time step after each pulse and after each resting interval, and the transient solution obtained at the end of each time step was used as the initial condition for the next time interval.

The electric field distribution was examined immediately after the first pulse and at the end of the pulse sequence. The values of both the electric field and the tissue temperature were stored over the time course of the simulation at designated locations in the ileum. In order to estimate the thermal damage experienced by the ileum, tissue temperatures at specific locations as well as the overall maximum temperature was stored directly after the completion of each pulse as well as once every second for three minutes after the last pulse in order to account for the entire thermal damage due to Joule heating affects [Maor and Rubinsky, 2010]. The maximum tissue temperature was used in order to gain a conservative estimate of the thermal damage that would be obtained, and the location-specific temperatures served to give a more accurate estimate of the thermal damage experienced in key areas throughout the tissue.

Thermal damage to biological tissues is dependent on both temperature and time, and the Arrhenius equation was used to quantify these effects [Tropea and Lee, 1992; Lee, 1991; Chang and Nguyen, 2004; Agah *et al*, 1994; Orgill *et al*, 1998; Lee and Astumian, 1996; Wright, 2003]. This equation was described in detail in Chapter 1 but, to summarize, this model uses Maxwell-Boltzmann statistics to describe how biological molecules at a temperature T are converted from a viable state to a thermally damaged state at a rate K [Lee, 1991]. This reaction can be described by a first-order chemical rate process [Agah *et al*, 1994]:

$$K = A \exp\left(\frac{-\Delta E}{RT}\right) = \frac{d\Omega}{dt} \quad (6.10)$$

Here R is the ideal gas constant, A is the measurement of molecular collision frequency, ΔE is the activation energy needed for the molecules to denature, t is the time, and Ω is the accumulated damage. The damage parameter Ω can be expressed as the logarithm of the relative concentration of the undamaged molecules at time zero and time τ :

$$\Omega(\tau) = \ln \left[\frac{C(0)}{C(\tau)} \right] \quad (6.11)$$

$C(0)$ and $C(\tau)$ are the amount of damaged and undamaged molecules at time zero and time τ , respectively. The Arrhenius equation given in Eq. 6.10 can be used to determine the Henriques and Moritz thermal damage integral:

$$\Omega(t) = \int A \exp\left(\frac{-\Delta E}{RT}\right) dt \quad (6.12)$$

The values of A and ΔE are based on experimental data. For this analysis, the parameters taken to be the same as those used in Chapter 5 ($A = 1.552 \times 10^{67} \text{ s}^{-1}$ and $\Delta E = 4.3 \times 10^5 \text{ J/mol}$) as given by Agah *et al.* and Wright [Agah *et al.*, 1994, Wright, 2003].

6.2.6 Parameters Modeled

For this model, the same electroporation parameters were used as modeled previously (Chapter 5) for a simplified, isotropic intestine model. These electrical parameters included 50 pulses with a pulse length of $70 \mu\text{s}$, an applied voltage of 200 V, and a pulse frequency of 4 Hz. In order to simplify the analysis, the electrical conductivities of muscle fibers measured parallel and perpendicular to the fiber orientation were used chosen for the muscle layers. Thus, $\sigma_{||} = 0.75 \text{ S/m}$, and $\sigma_{\perp} = 0.135 \text{ S/m}$ [Corovic *et al.*, 2010]. Though these values are likely to vary from the true electrical conductivities of the muscle layers of the small intestine, they were chosen as an approximation in order to investigate how the electric field distribution depended on the heterogeneous nature of the tissue. The electrical conductivities of the remaining layers were kept at a uniform electric conductivity (σ_t) and were varied from 0.1 S/m to 0.8 S/m.

6.3 RESULTS

By taking into account the changes in electrical conductivity with different tissue layers, the heterogeneous effect of the small intestinal tissue on the resulting electrical field can be seen. These results are shown for the case in which the electrical conductivity of the inner layers (σ_t) is set at 0.6 S/m. The electric conductivity distribution and corresponding electric field distribution that occurs during an electrical pulse are shown in Figure 6.8.

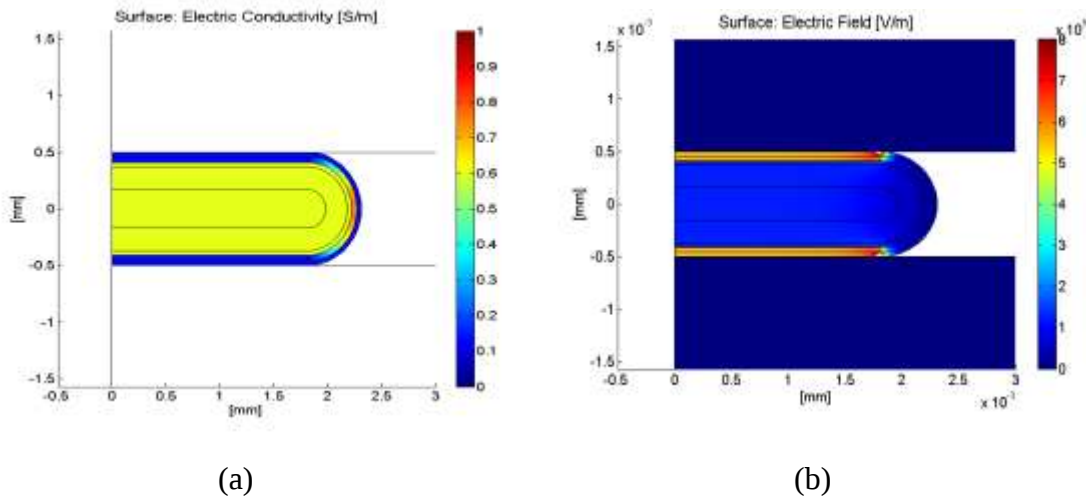


Figure 6.8 Electrical conductivity and electric field distribution. Here, the surface electrical conductivity (a) and the resulting electric field distribution (b) are shown for the scenario in which $\sigma_t = 0.6 \text{ S/m}$.

As can be seen, the lower electrical conductivity of the muscle layers results in a higher electrical field in these areas. It can also be noted that the electric field spikes due to edge effects at the corners where the small intestine tissue first meets the electrodes. Nine specific locations were chosen for further evaluation of electric field magnitude and thermal effects. These points are illustrated in Figure 6.9 and their corresponding coordinates are given in Table 6.3.

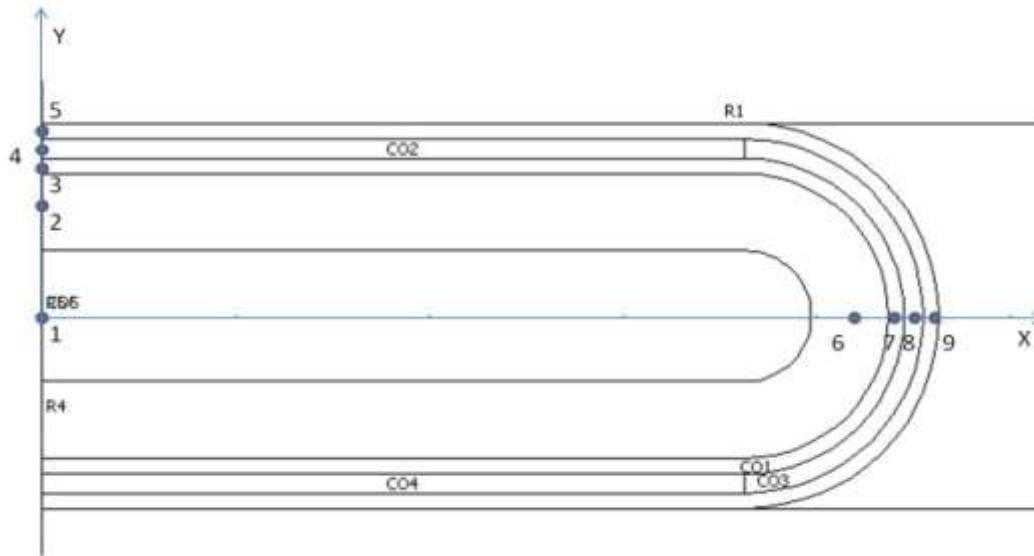


Figure 6.9. Locations chosen to analyze the local electrical and thermal effects. The red dots mark specific locations on the small intestine model geometry where local electric field magnitudes and thermal effects were further examined.

Table 6.3. Location coordinates. The (x,y) coordinates for the specific locations illustrated in Figure 7.9 are given in units of millimeters.

1	2	3	4	5	6	7	8	9
(0,0)	(0, 0.27)	(0, 0.39)	(0, 0.435)	(0, 0.48)	(2.085, 0)	(2.205, 0)	(2.25, 0)	(2.295, 0)

Table 6.4 gives the electric field magnitude at each of the locations illustrated in Figure 6.9 for a range of inner layer tissue electrical conductivities (σ_t : 0.1 – 0.8 S/m).

Table 6.4. Electric field magnitude. The magnitude of the electric field at each location is given in units of kV/cm. Here, the electric field can be compared for the different electrical conductivities modeled. The gray background highlights locations of highest electric field magnitude.

σ_t [S/m]	1	2	3	4	5	6	7	8	9
0.1	2.0979	2.0979	2.0979	1.554	1.554	0.8818	0.5977	0.6649	0.5435
0.2	1.8405	1.8405	1.8405	2.7267	2.7267	0.7374	0.4967	0.5529	0.4517
0.3	1.6393	1.6393	1.6394	3.6431	3.6431	0.6368	0.4277	0.4763	0.389
0.4	1.4777	1.4778	1.4779	4.3789	4.379	0.5617	0.3765	0.4195	0.3424
0.5	1.3452	1.3453	1.3453	4.9828	4.9828	0.5029	0.3368	0.3752	0.3063
0.6	1.2344	1.2345	1.2346	5.4873	5.4873	0.4556	0.3049	0.3397	0.2773
0.7	1.1405	1.1406	1.1407	5.9151	5.9151	0.4166	0.2786	0.3105	0.2534
0.8	1.0599	1.06	1.0601	6.2824	6.2824	0.3838	0.2566	0.286	0.2334

Locations 4 and 5 resulted in the highest electric field values for all values of electrical conductivity modeled except for $\sigma_t = 0.1$ S/m. Thus, location 5 at (0, 0.48 mm) was chosen to compare the temperatures experienced due to the range of electrical conductivities. Figure 6.10 illustrates how temperature accumulation increases with the electrical conductivity value. The temperature obtained at the end of each pulse over the 50 pulse protocol is shown as well as the temperature after the pulse is removed.

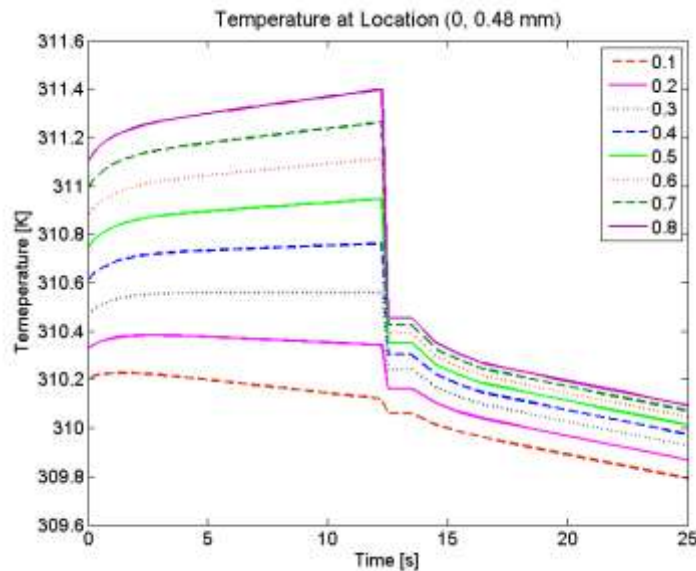


Figure 6.10. Local temperature at location (0, 0.48 mm). The local temperature obtained during the pulse procedure and after the pulse is removed is compared for the range of inner layer electrical conductivities modeled. Here, the electrical conductivity values shown in the legend are given in units of S/m.

The temperature obtained over the first 8 pulses is shown in Figure 6.11. As can be seen, the local temperature spikes up sharply during the applied electrical pulse but drops quickly between pulses, helping to minimize thermal effects within the tissue.

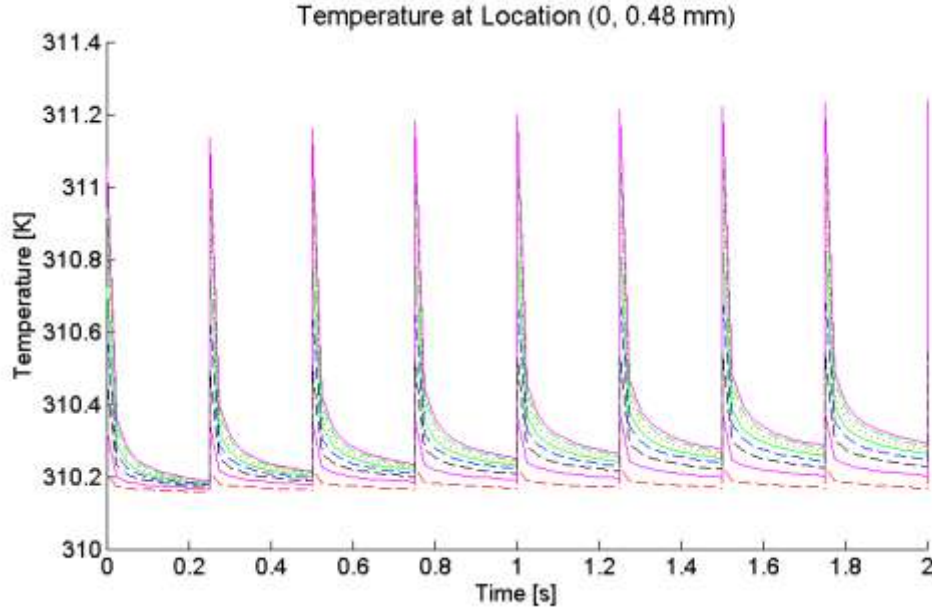


Figure 6.11. Local temperature during pulses. The temperature obtained at location (0, 0.48 mm) is given for the range of inner layer electrical conductivity (0.1 – 0.8 S/m) over the course of the first 8 pulses. Here, the lower red dotted line corresponds to an electrical conductivity of $\sigma_t = 0.1$ S/m, and the thermal effects increase with electrical conductivity up to the top purple line of $\sigma_t = 0.8$ S/m.

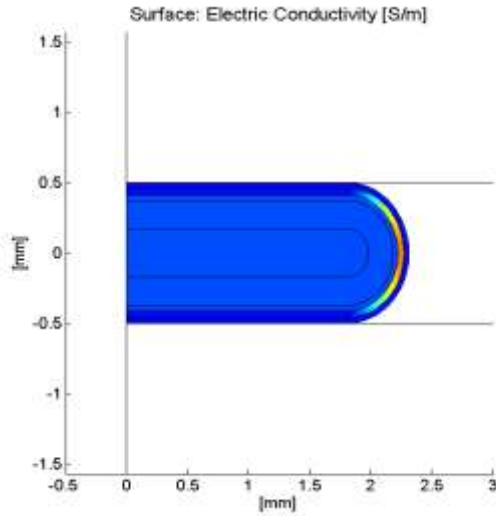
The damage parameter was calculated using temperatures at the location of (0, 0.48 mm) as well as using the maximum temperature obtained throughout the simulation. In both cases, the largest damage parameter occurred when σ_t was the highest (0.8 S/m). Nonetheless, even then, the damage parameter stayed relatively low, resulting in a local damage parameter of $\Omega_{\text{local}} = 6.7 \times 10^{-4}$ and a damage parameter corresponding to the maximum temperature obtained throughout the simulation of $\Omega_{\text{max}} = 0.0037$ S/m. This gives an estimated damage of 0.07 % and 0.37 %, respectively.

6.4 DISCUSSION

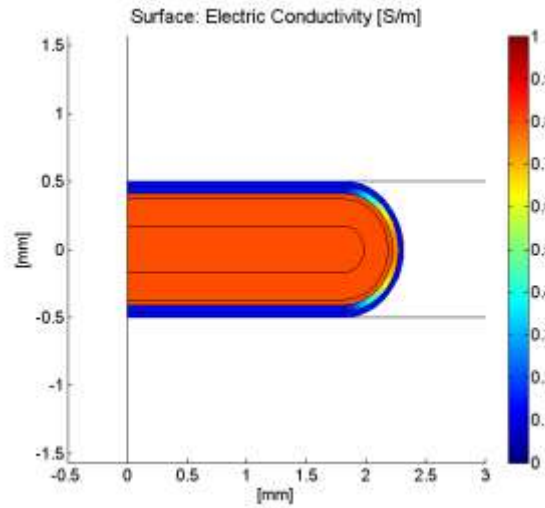
Here, the heterogeneous effect of the tissue layers was investigated specifically for the rat small intestine geometry. As shown here, this heterogeneous effect strongly affects the electric field distribution in the tissue. Since values could not be found in literature for the electrical

conductivities for each specific small intestinal layer, the inner layers of the small intestine were varied from 0.1 – 0.8 S/m, and the electrical conductivities for the muscle layers were taken as 0.135 S/m for muscle fibers that lay perpendicular to the applied electric field, and 0.75 S/m for muscle fibers oriented parallel to the electric field. It is evident that when the muscle layers near the electrodes have a lower electrical conductivity than the inner layers of the small intestine, the electric field becomes concentrated within the muscle layer, resulting in high electric fields and greater thermal effects in the outer layers and a much lower electric field within the inner layers. This is shown in Figure 6.8b, where the inner layers of the small intestine are modeled as having an electrical conductivity of 0.6 S/m as compared to the lower muscle electrical conductivity of 0.135 S/m.

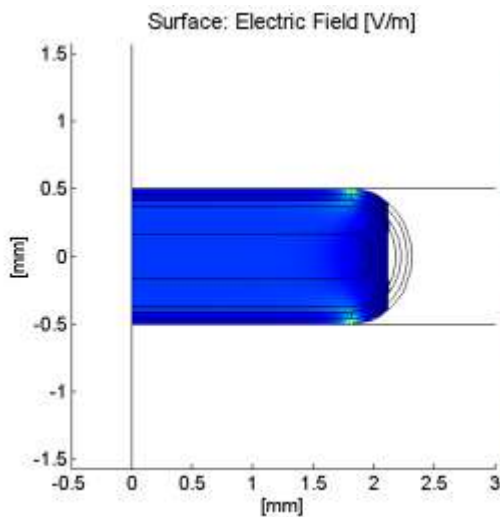
This effect is further illustrated by comparing the two extreme cases when $\sigma_t = 0.1$ S/m to when $\sigma_t = 0.8$ S/m. This is illustrated in Figure 6.12. As can be seen, when the lower electrical conductivity value is used, a more uniform electric field distribution results throughout the small intestinal layers. Increasing the electrical conductivity, however, results in a large difference in electrical conductivity between the outer muscle layers and the inner layers.



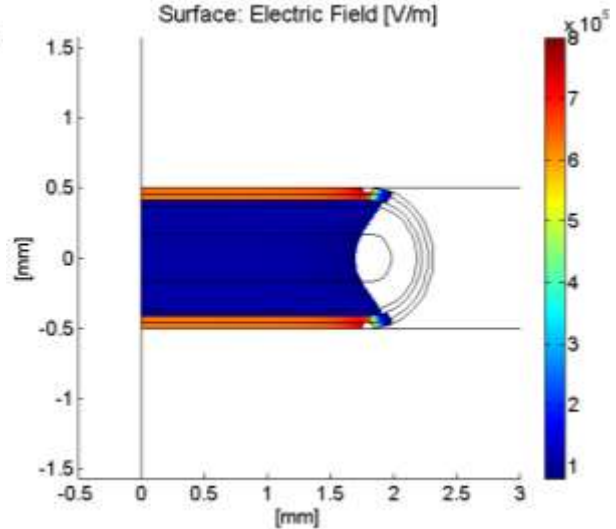
(a)



(b)



(c)



(d)

Figure 6.12 Comparing electric field distributions for $\sigma_t = 0.1$ S/m to the case when $\sigma_t = 0.8$ S/m. The electrical conductivity distributions are shown for the lower electrical conductivity case (a) and for when the inner intestinal layers are modeled as having an electrical conductivity of 0.8 S/m (b). The resulting electric field distributions are given in (c) and (d), respectively. As can be seen, an electrical conductivity of 0.1 S/m results in a much more uniform electric field distribution (c) as compared to the very layer-dependent electric field distribution that occurs due to an inner layer electrical conductivity of 0.1 S/m (d). Here, the electric field is only shown for magnitudes above 800 V/cm. As can be seen, the higher electrical conductivity case results in less of the small intestinal tissue experiencing an electric field above 800 V/cm. In either case, however, the curved end of the small intestine does not have an electric field above this value.

In Figure 6.12, the electric field is shown for values above 800 V/cm. The irreversible electroporation threshold report in literature is around 800 V/cm [Davalos et al, 2005]. Since the exact threshold for irreversible electroporation depends on the specific tissue as well as the electric pulse duration and number and since these values have not yet been determined experimentally for the small intestine, a value of 800 V/cm was used here to represent when this threshold may occur. This model predicts that for the entire range of electrical conductivities modeled, the curved end of the small intestine will experience an electric field below this threshold value, possibly resulting in sections of the small intestine that would not experience cell ablation by electroporation.

The thermal effects were also examined at specific locations on the small intestine model geometry. As illustrated in Figures 6.10 and 6.11, higher temperatures are obtained for the case in which the inner electrical conductivity (σ_i) is also higher. The location of maximum temperature occurred in the muscle layers near the electrodes for all cases except when σ_i was less than the muscle electrical conductivity. This corresponds to the increase in electric field magnitude in the muscle layer. Through it was seen that temperature does correspond to the value of tissue electrical conductivity, the temperature did not change much for any of the scenarios modeled, resulting in an increase of less than 1.5 °C during the pulse protocol. This very small change in temperature can be partially contributed to the large stainless steel electrodes that help conduct heat away from the small intestine.

The accumulated thermal damage was also measured at each specific location in the small intestine geometry. The maximum thermal damage corresponded to the location of highest electric field, resulting in approximately 0.07 % damage. However, despite the fact that the majority of the small intestine experiences very small changes in temperature that do not account for any significant thermal damage, it is possible that some thermal damage could occur at the singularity points where the curved section of the small intestine meets the electrodes. Here, these singularity effects cause a large spike in the electric field, which could potentially lead to thermal damage in the immediate vicinity. Nonetheless, using the maximum temperature obtained throughout the simulation resulted in thermal damage of approximately 0.37%. Thus, though some thermal damage may occur immediately around the singularity point, this model indicates that heat is able to be conducted away from the tissue very quickly due to the large stainless steel electrodes, and thus, any potential damage to the small intestinal tissue is kept at a minimum.

This model indicates that it is important to take into account the heterogeneous layers of tissues such as the small intestine when predicting electric field response for irreversible electroporation. Future work would include adding additional factors to this model. For example, most tissues have been shown to have an electrical conductivity that depends on temperature with a positive temperature coefficient for electrical conductivity between 1 and 3 % $^{\circ}\text{C}^{-1}$ [Duck, 1990]. However, since this model predicts very small temperature increases, it is expected that adding these temperature dependence effects into the model would not significantly affect the result, and thus these effects were neglected.

Currently, electrical conductivity values for the small intestine layers have not been measured. The model described here shows that for a range of possible values, the electric field

distribution can vary drastically. Thus, knowing these properties is essential for developing a more accurate model. In addition, it has been shown experimentally that the electrical conductivity of tissue changes once the tissue becomes permeabilized. This is a threshold phenomenon that results in an increase in tissue electrical conductivity. For small intestine models where the inner layers had a higher electrical conductivity than the outer muscle layers (for example: $\sigma_t = 0.8$ S/m shown in Figure 6.12d) the electric field was seen to have a much higher magnitude in the outer muscle layers. By taking into account changes in tissue electrical conductivity, however, it is likely that a more uniform electric field distribution would occur. Once the outer muscle layers reached the threshold for electroporation, the electrical conductivity of these layers would increase, allowing the electric field in the inner layers to increase. Data on the changes in electrical properties of biological tissue after electroporation are very scarce, and currently the irreversible electroporation threshold is only available for limited pulse parameters for a few different types of tissues [Zupanic and Miklavcic, 2010]. Thus, before these effects can be taken into account for modeling the effects of electroporation, it is first necessary that the electrical conductivity values and how they change during electroporation are determined experimentally. Once these values are known, a more in depth model can be developed that better predicts how the electric field distribution develops on heterogeneous tissues.

6.5 CONCLUSIONS

Here it is shown that in order to more accurately predict the electric field distribution in heterogeneous tissues due to electroporation, each tissue layer must be modeled. Changes in tissue electrical conductivity from layer to layer in the small intestine were shown here to cause substantial changes in the electric field distribution. Though this model supports the results from the homogenous intestine model in Chapter 5 that most of the small intestinal tissue will avoid thermal damage effects, it shows that singularities occur where the edges of the electrodes meet the tissue, and this could cause small local areas of heating. In addition, for the range of electrical conductivity values analyzed, this model predicts that a small portion of the intestine may not experience an electric field strong enough to cause irreversible electroporation to occur. Currently, experimental data cannot be found in the literature for the electrical conductivity values for each layer of the small intestine tissue, and the change in electrical conductivity due to the occurrence of electroporation is also unknown. Here, it is shown that these values are essential for developing a more in-depth heterogeneous tissue model of electroporation. Future work would include experimentally determining these parameters, adding them to the model described here, and also accounting for the increase in electrical conductivity due to electroporation. Further development of this model is essential for treatment planning of irreversible electroporation when used to treat tumors that are embedded in or adjacent to heterogeneous tissues such as the small intestine.

CHAPTER 7: NTIRE LEADS TO SMALL INTESTINE RECOVERY IN VIVO

7.1 INTRODUCTION

Non-thermal irreversible electroporation (NTIRE) has recently been conceived as a new minimally invasive ablation method, using millisecond electric fields to produce nanoscale defects in the cell membrane bilayer and induce cell death while keeping all other molecules, including the extracellular matrix, intact. One emerging application of NTIRE is in relation to treatment of abdominal cancer and the ability to avoid collateral damage even in tissues within the electric field. In this work the focus is on studying the effect of the molecular selectivity of NTIRE on a body organ that is very often subject to collateral damage in minimally invasive or non-invasive surgery: the small intestine. For instance, collateral damage to the small intestine often occurs after radiotherapy for pelvic or abdominal malignancies as well as a side effect of chemotherapy, resulting in bloating, abdominal cramping, severe diarrhea, nausea, and vomiting [Han *et al*, 2011; Keefe *et al*, 2000; Ciorba and Stenson, 2009]. These side effects are seen as the limiting factor in increasing both chemotherapy and radiotherapy dosage and can force discontinuation of treatment [Keefe *et al* 2000; Packey and Ciorba, 2010]. The small intestine may be especially susceptible to these treatment methods since it experiences a high cell turnover rate, especially for the rapidly dividing cells of the mucosa [Keefe *et al*, 2000]. The hypothesis is that, due to the molecular selectivity of NTIRE and its ability to spare the extracellular matrix, the intestine will remain structurally intact after treatment with NTIRE, survive the treatment, and recover. This study was performed in a small animal model in which effects of applying a typical NTIRE protocol directly to the intestine were studied.

Here, the first *in vivo* study is presented that examines the effects of NTIRE on the small intestine, an organ whose collateral damage is of particular concern in the anticipated use of NTIRE for treatment of abdominal cancers. The NTIRE electrical parameters that were chosen from the analysis described in Chapter 5 were applied directly to the rat small intestine. Histological analysis was used to then examine the effects of NTIRE over time.

7.2 METHODS

Twelve Sprague-Dawley rats weighing 200-300 g were used in this study. All animals received humane care from properly trained professionals in compliance with both the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (NIH publication No. 85-23, revised 1985).

Animals were anesthetized with 2 mg/kg meloxicam followed by chamber induction with isoflurane. Anesthesia was administered throughout the procedure with vaporized isoflurane. The depth of anesthesia was assessed prior to surgery and throughout the surgical procedure. After the level of anesthesia was verified, the abdominal skin was shaved and an antiseptic was applied. Lidocaine (up to 7 mg/kg) was administered subcutaneously along the midline of the abdomen as a local anesthesia. A 3-cm midline abdominal incision was made, exposing the small intestine. A set of plate electrodes (BTX Caliper Electrode, Harvard Apparatus, Holliston,

MA) was gently applied across the ileum, about 5 cm proximal to the ileocecal valve. The measured distance between the two electrodes was approximately 1 mm and was consistent for all animals tested. A sequence of 50 DC pulses of 200 V (corresponding to an electric field of approximately 2000 V/cm), 70 μ s each, and a frequency of 4 Hz was applied between the electrodes using a high voltage pulse generator designed for electroporation procedures (ECM 80, Harvard Apparatus, Holliston, MA). The electrical parameters used in this study are typical of those used in clinical procedures to produce irreversible electroporation without causing thermal damage to the intestinal tissue and were chosen based on finite element analysis of the resulting electrical and thermal effects (Chapter 5). The procedure was repeated in two successive locations along the ileum, treating approximately 2 cm along the length. The location of treatment was noted based on anatomy, and a suture knot was placed in the mesentery to mark the IRE-treatment zone. At the end of the experiment, the abdomen wall was sutured closed, followed by the skin incision. Tissue adhesive was applied over the skin sutures, and wound clips were placed on either side of the suture in order to distract the rat from grooming its sutures. Buprenorphine (0.05 mg/kg) was administered as an analgesic following the procedure. Animals were divided into three groups of four animals each and were kept alive for one, three, or seven days prior to being euthanized.

During the first 24 hours after surgery, the animals were given two additional doses of buprenorphine (0.05 mg/kg) and meloxicam (2 mg/kg), spaced out over 8 hour increments. After surgery, animals were checked daily to ensure that they recovered, stayed healthy, and were not experiencing pain. Symptoms that were monitored included reduced food intake, fever, hunched posture, lack of grooming or locomotion, swelling around the incision, facial discharges around the nose and eye, and diarrhea. All animals were also weighed daily.

Grooming of the sutures was a problem with this procedure, since the animal could very easily reach its incision with its teeth. Since male rats tend to groom less than female rats, male animals were predominately used. As an additional precautionary measure, an Elizabethan collar was built such that the animal could still eat and drink normally, but would not be able to reach and groom the sutures. The collar and dimensions, designed for a 250 – 300 g rat, are illustrated in Figure 7.1. The collar was attached in a conical fashion around the animal's neck, loose enough to not cause discomfort, but tight enough such that the rat could not pull the collar off. The collar kept the rat from using its teeth to groom around its incision and pull out the sutures prematurely.

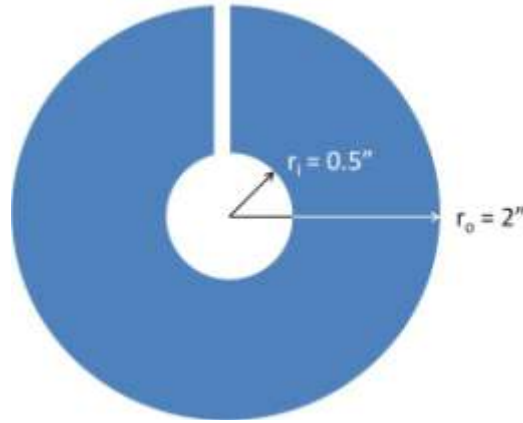


Figure 7.1. Elizabethan collar designed to protect animal's incision after surgery during healing stage. The collar was secured around the rat's neck, allowing it to maintain daily activities, but preventing the animal to groom around its incision.

Animals were euthanized by bilateral chest dissection while under a deep anesthesia induced by vaporized isoflurane and an intraperitoneal injection of ketamine (90mg/kg) and xylazine (10 mg/kg). The treated regions of the small intestine as well as untreated sections 3-5 cm proximal and 3-5 cm distal of the treated region were harvested. Each intestinal segment was flushed with saline, fixed with 10% buffered formalin, and submitted to an independent pathology lab (Pathology Associates, Inc., Berkeley, CA). The samples were embedded in paraffin and sectioned with a microtome (5- μ m-thick). All samples were cut perpendicular to the intestinal axis, exposing the ileum's cross section. Each sample was stained with hematoxylin and eosin (H&E). Select samples from each group were cut in cross section and stained with Masson's trichrome to examine the structure of the extracellular matrix.

Examination of each section was focused on the small intestine's cellular and extracellular response to NTIRE over time.

7.3 RESULTS

Thirteen Sprague-Dawley rats were used in this study. One animal was lost during surgery due to an overdose of isoflurane. All other animals recovered quickly from the surgical procedure and remained active, maintaining weight over the one to seven day period. Normal eating habits and stool were observed. Five of the animals experienced slight porphyrin staining around the eyes after surgery that cleared up on its own within 24 hours. Otherwise, the animals did not display any of the typical signs of pain, and observations indicated that the animals did not experience any adverse effects due to the NTIRE-treatment procedure.

Histological analysis of the small intestine 1 day, 3 days, and 7 days was used to examine the effect of NTIRE on the small intestine over time. At Day 1, ileum segment exhibited severe necrotic tissue with complete obliteration of cellular architectural details. At 3 days after treatment, the structure of the small intestine was still necrotic. At 7 days, however, the ileum

appeared to have regained much of its structure and showed distinct tissue layers such as the mucosa, submucosa, muscular layers, and serosa. This can be seen in Figure 7.2.

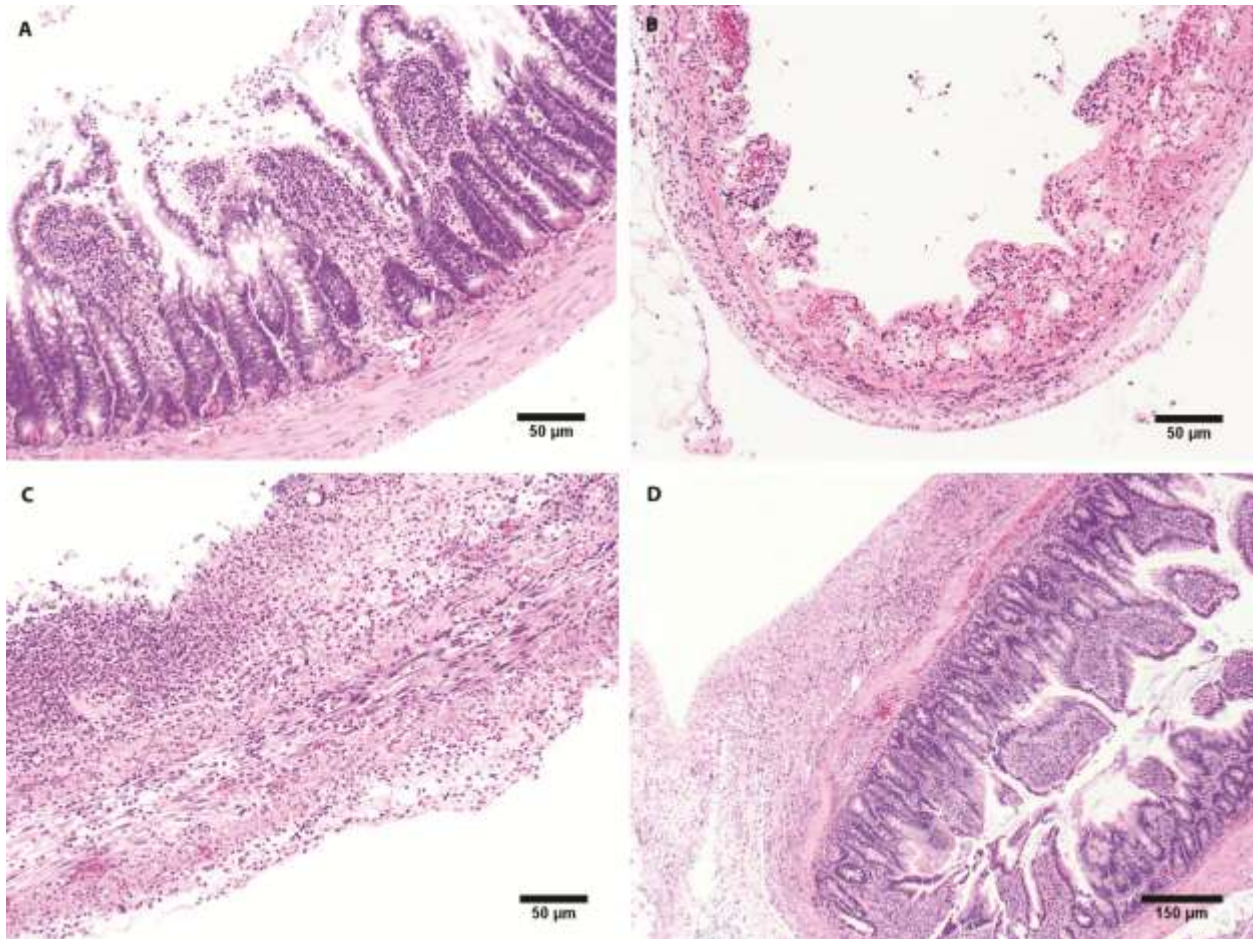


Figure 7.2. The effect of NTIRE on the small intestine 1, 3, and 7 days after NTIRE-treatment. (a) The untreated control shows a typical, healthy small intestine. (b) One day after NTIRE-treatment, the small intestine shows complete cellular ablation. (c) Treated areas 3 days after treatment still depict a loss in the structural layers of the cell. (d) At 7 days after applying the NTIRE protocol to the small intestine, the distinct structure of the small intestine is seen.

The results 1 day after NTIRE-treatment (Fig. 7.2) show that the irreversible electroporation protocol was strong enough to affect all layers of the small intestine. Here, a complete loss of cellular architectural detail can be seen, and the villi are losing organization and form. Though acute necrotic tissue was observed along the entire circumference of the NTIRE-treated regions, no perforations were observed, indicating that the structure of the small intestine was still intact enough to keep fissures from forming and the luminal contents from spilling outward.

The original villi is completely obliterated at 3 days after applying NTIRE (Figs. 7.2 and 7.3). Here, though the extracellular structure still exists, the tissue is void of the proper cellular structure and tissue layers. Signs of tissue repair, however, are evident. A new epithelial layer can be seen forming along the edges of the treated zones, as indicated by the appearance of immature epithelial cells. In addition, blood vessels and nerve bundles are present and regenerating myocytes can also be seen.

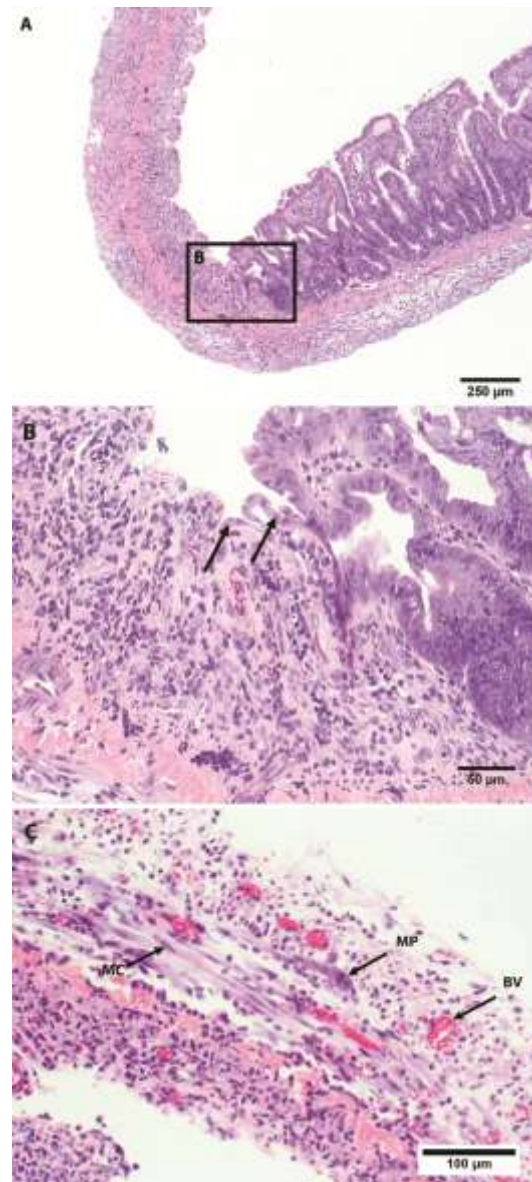


Figure 7.3. Small intestine 3 days post-NTIRE. (a) The interface between an NTIRE treated region and an untreated region of the small intestine is shown. (b) A closer look with higher magnification reveals immature endothelial cells that can be seen migrating into the NTIRE-treated zone, as highlighted by the arrows. (c) The presence of blood vessels (BV), the myenteric plexus (MP), and myocytes (MC) can also be seen.

At 7 days post-NTIRE, the tissue structure appears to have recovered into its distinct layers (Fig. 7.4). The mucosa is in the process of organization, and normal repair and replacement is occurring. The immature, frond-shaped villi are lined with epithelial cells, and immature muscle cells are now present in the muscle layers. Regenerating granular cells are also present.

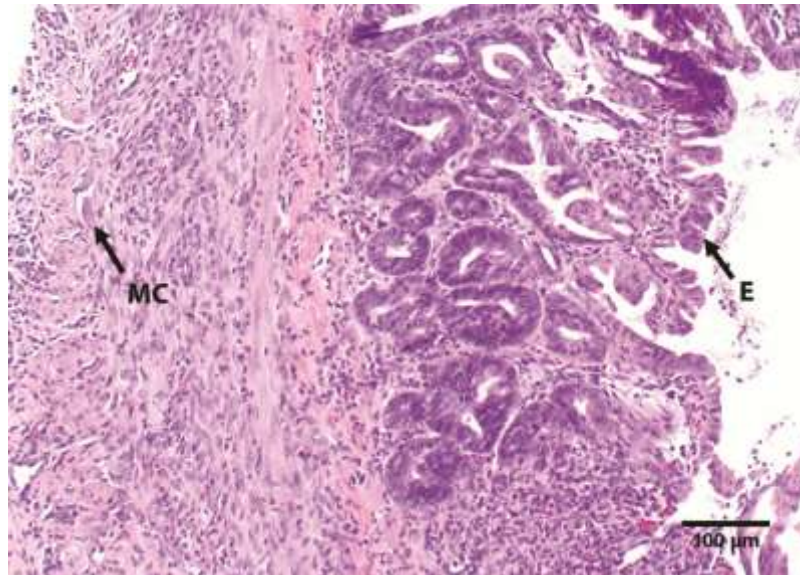


Figure 7.4. Small intestine 7 days post-NTIRE. The small intestine is beginning to regain its cellular structure 7 days after NTIRE-treatment and the mucosa has regenerated, as indicated by the presence of new villi lined with epithelial cells (E). The muscularis is also becoming repaired with immature muscle cells (MC).

Masson's trichrome stain was also used on select intestinal samples in order to examine the effect of NTIRE on the extracellular matrix. Here, an NTIRE-treated sample harvested 1 day after the procedure is compared to the control (Fig. 7.5). The collagen fibers are stained blue, muscle fibers are stained red, and cell cytoplasm and nucleus are stained light red and dark brown, respectively. Though the cellular makeup of the intestinal tissue is strongly affected by the NTIRE-treatment procedure, it can be observed that the extracellular makeup is very similar in morphology between the treated and untreated samples, indicating the extracellular architecture is still intact.

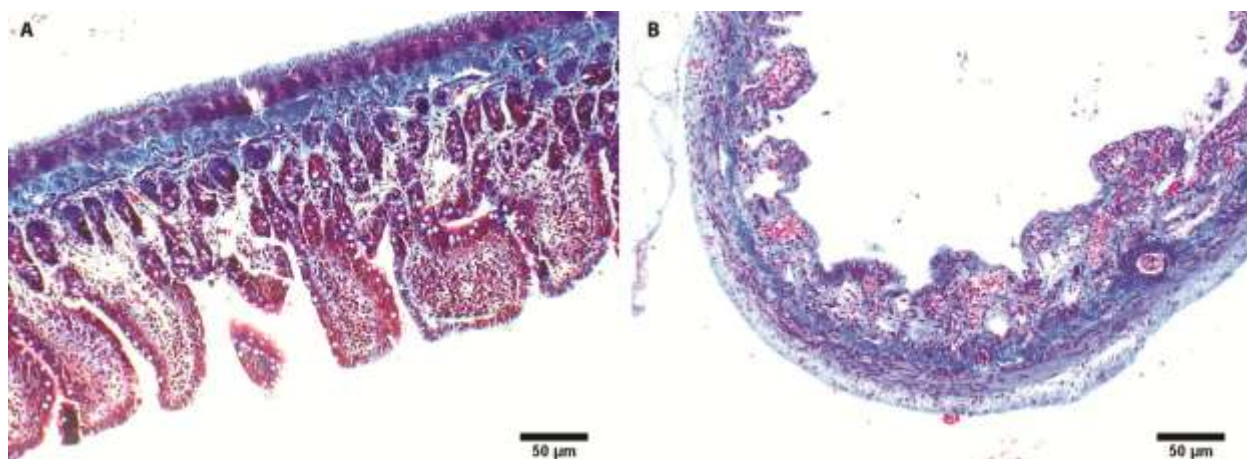


Figure 7.5. Effect of NTIRE on cell scaffold structure. Although 1 day after NTIRE-treatment, there is a loss of cellular architecture throughout the intestine (b) as compared to the control (a), the cell scaffold remains intact. The blue collagen fibers are similar in morphology after NTIRE treatment when compared to the control.

7.4 DISCUSSION

In this study, a typical NTIRE electrical pulse protocol was applied across the small intestine, in order to assess the tissue's ability to respond and recover to the NTIRE treatment. Though the plate electrodes used in this study are not the method of applying NTIRE clinically, they are convenient for inducing a pulsed electric field across the tissue in a lab setting, enabling one to study the direct effect of the electrical protocol on the tissue's ability to recover. At one day after treatment, the small intestine saw complete cellular ablation throughout its entire circumference, indicating that the electrical parameters chosen were strong enough to cause irreversible electroporation throughout all layers of the tissue. For this study, we wished to provide complete damage to the tissue by electroporation while avoiding any effects of thermal damage. Using finite element modeling (as described in detail in Chapter 5), the electrical parameters were chosen such that they would be well above the threshold for irreversible electroporation without resulting in thermal damage due to Joule heating to the tissue. As is evident in Figure 7.2, the cellular destruction to the tissues is complete and a full loss of cellular architectural detail can be seen. However, since the extracellular matrix is not affected by NTIRE, the structural integrity of the small intestine remained.

Though some samples saw complete obliteration around the entire circumference three days after treatment, Figure 7.3a indicates a case in which a section along the circumference of the small intestine shows cell ablation that is immediately adjacent to a section that was not affected by the electroporation protocol. The heterogeneous modeling described in Chapter 6 predicted that the curved sections of the small intestine, when pressed between the two electrodes, experienced a lower electric field than the rest of the tissue. The histological results shown here indicate that, some cases, this may have been the case and the electric field may have dropped below the threshold needed for electroporation, resulting in the appearance of untreated

intestinal tissue, as seen in Figure 7.3a. Nonetheless, in all cases, complete obliteration of the cellular structure occurred after electroporation around the majority of the circumference, and these irreversibly electroporated sections were able to be further examined over time.

Despite the complete obliteration of the cellular structure, the tissue showed signs of recovery. The modality of cell death due to NTIRE occurs quickly [Lavee *et al*, 2007], and though the small intestine villi and crypt were completely destroyed, signs of tissue repair are already evident three days post-treatment (Fig. 7.2). The crypts contain multipotent stem cells which differentiate and move up the villi, replacing cells that slough off in normal, healthy tissue every 1-3 days [Ciorba and Stenson, 2009; Dignass, 2001]. Though the cells within the crypts are ablated within the treated area, it appears that immature epithelial cells are being produced from the edges of the treated zones and are able to migrate inward, producing a new epithelial cell layer. In addition, it can be seen that the framework of the muscularis is preserved at both 3 days and 7 days after NTIRE (Figs. 7.3 and 7.4). Tissue recovery continues at 7 days post-NTIRE, where repair is evident and the tissue appears to have regained its distinct layers (Fig. 7.4). Normal repair and replacement of the mucosa, submucosa, and muscularis is occurring. Though additional studies are needed in order to assess tissue function and investigate the effects of NTIRE on the intestine over a longer time course than 7 days, it is evident here that the small intestine was able to go from complete cellular destruction to regeneration of intestinal layers and villi within one week. Longer-term studies are planned in order to assess the continued recovery of the small intestine.

NTIRE specifically targets the cell membrane, allowing for the preservation of tissue structural components such as the extracellular matrix, blood vessels, and nerves [Phillips *et al*, 2010; Onik and Rubinsky, 2010]. It can be seen that this holds true for the small intestine as well. Masson's trichrome staining of the ileum at one day after NTIRE treatment illustrates that the extracellular matrix is still intact (Fig. 7.5). Lymphatic supplies, nerves, and blood cells are still functioning, providing a framework for epithelialization that can be observed at 3-days post NTIRE-treatment. This framework allows for restoration of the blood supply, as seen for the 3-day and 7-day treatment groups. Thermal coagulation and thrombosis to the blood vessels has not occurred, and the capillaries are open and blood is flowing (Fig. 7.3), resulting in presence of immature villi and granular cells seven days after electroporation. It is hypothesized that the ability of NTIRE to preserve important structures such as the extracellular matrix, blood vessels, and nerves greatly aids in the overall recovery of the small intestine.

As illustrated both here and in the literature [Rubinsky, 2007; Onik and Rubinsky, 2010], NTIRE preserves the tissue vasculature, as compared with the vascular damage that can result from ionizing radiation [Packey and Ciorba, 2010]. Vascular damage occurs during ionizing radiation [Demirer *et al*, 2007], and some believe that this damage can lead to complications with the small intestine years after treatment [Packey and Ciorba, 2010; Paris *et al*, 2001]. Though NTIRE does cause endothelial cell death, vessel occlusion does not occur [Onik and Rubinsky, 2010], and endothelial cells have been shown to reline the blood vessels within a week of NTIRE treatment [Phillips *et al*, 2011], leaving an intact and functioning micro and macro vasculature. Though long term studies would be needed in order to determine what effects NTIRE has on the small intestine years after treatment, it is believed here that the unique ability of NTIRE to preserve blood vessels and extracellular matrix not only aids in short term

recovery, but could also protect the tissue from developing the long term complications often seen from radiation treatments.

NTIRE is viewed as a promising modality for cancer treatment. Due to its ability to preserve important structural and functional aspects of the tissue while specifically targeting the cell membrane, NTIRE may be a promising alternative for treating malignant tumors located near sensitive organs. For example, ablating abdominal tumors could cause damage to small intestine. The goal of this study was to evaluate the ability of the small intestines to survive direct application of NTIRE. For this study, 2000 V/cm were applied directly to the small intestine, resulting in complete cellular ablation one day after treatment. The extracellular matrix, blood vessels, and nerves, however, were preserved, aiding in recovery of the tissue. By three days after treatment, the endothelial layer had begun to recover, and the 7-day group showed regeneration of the villi and a restored structural layer including the mucosa, submucosa, and muscularis. Although substantial further investigation is needed, this pilot study indicates that the high turnover rate of the small intestine mucosa coupled with the molecular selectivity of NTIRE and its ability to preserve the extracellular matrix and other important functional structures allows for a quick recovery of the intestine after electroporation treatment. This study predicts that, should the small intestine be within the electric field generated while treating an abdominal tumor with NTIRE, the intestine will be able to heal and regenerate.

7.5 CONCLUSION

Theoretical, mathematical, and biophysics principles have recently led to the conception of a new minimally invasive surgery that is molecularly selective, producing only nanoscale defects in the cell membrane bilayer to induce cell death while keeping all other molecules, including the extracellular matrix, intact. This technology uses strong millisecond electric fields and is referred to as non-thermal irreversible electroporation (NTIRE). Here, the theoretical claim of molecular selectivity on more firm experimental grounds. This is the first *in vivo* study that explores how the molecular selectivity affects the application of NTIRE on the small intestine, an organ whose collateral damage is of particular concern in the anticipated use of NTIRE for treatment of pancreatic cancer. The small intestine has shown susceptibility in other treatment methods, and it is often the limiting factor in conventional minimally invasive surgeries, such as radiation therapy. A typical NTIRE electrical protocol was applied directly to the rat small intestine. It was shown here that the molecular selectivity of NTIRE led to the complete ablation of the cells in targeted tissue. However, since the extracellular scaffold remained intact, the animals did not show any physiological effects of the procedure and the intestine was able to recover, completely developing an epithelial layer at 3 days post-treatment and regenerating mucosa, submucosa, and muscular layers within a week. This novel procedure can be utilized for abdominal cancer treatment while minimizing collateral damage to adjacent tissues due to the unique molecular selectivity of the NTIRE ablation method.

CHAPTER 8: DISSERTATION SUMMARY AND FUTURE WORK

8.1 DISSERTATION SUMMARY

Non-thermal irreversible electroporation (NTIRE) is a very promising method for tissue ablation in medical applications such as cancer treatment. Indeed, NTIRE has seen success in clinical trials for tumor ablation, including the treatment of prostate cancer and cancer in the kidneys. NTIRE utilizes a series of microsecond electrical pulses that target the cell membrane, causing pores to form and leading to non-thermal cell death. Though this treatment methodology has thus far been seen as very successful and having great potential, there are still many areas of further research to be investigated before NTIRE can be fully harnessed for tumor ablation as well as other medical applications. One important area is examining how NTIRE affects critical tissues that may experience electroporation due to their proximity to the targeted tumor. Here, the artery and the small intestine were examined as two potential tissues whose recovery and continued function is essential after NTIRE treatment. In addition, the results obtained from applying NTIRE to the artery were also examined in the context of developing a method to decellularize tissues for use as a natural tissue scaffold. Here, the direct effect of NTIRE on both the artery and the small intestine was examined. This is important in not only gaining a more in depth understanding of the time scale and process of tissue recovery after NTIRE but it is also essential in designing treatment plans for tumors within the vicinity of critical tissues.

8.1.1 Effect of NTIRE on the Artery

8.1.1.1 Artery Recovery for Cancer Treatment Applications

In order to gain a deeper understanding of how NTIRE affects the artery's ability to recover and function after treatment, experimental protocols were developed to apply NTIRE *in vivo*, examining the artery's recovery over time. First, finite element analysis was utilized to predict the resulting thermal and electric fields in order to choose an electroporation protocol that could cause irreversible electroporation to the tissue while avoiding thermal damage. The solution for two parallel plate electrodes applying NTIRE across the outside of the artery was compared to that developed by Maor and Rubinsky [Maor and Rubinsky, 2010] using an endovascular device to apply NTIRE from the artery lumen in a minimally invasive manner. Though the endovascular device may be more clinically relevant for cases when it is desired to apply NTIRE to the artery directly, the parallel plate electrodes are much more applicable for the laboratory setting. For both electrode devices, electroporation parameters were chosen that would cause minimal thermal damage to the tissue. These electroporation parameters were then used *in vivo*, applying NTIRE directly to the rat carotid artery using the plate electrode, and comparing these results to those obtained by applying NTIRE to the rabbit iliac artery using the endovascular electrode device.

The artery's recovery was examined over a one week post-treatment time period. It was seen that for both types of treatment, the artery became naturally decellularized between 3 and 5 days after applying NTIRE. By seven days after treatment, however, the artery had already begun to recover, developing a full endothelial cell layer. In addition, it was seen that the

structure of the extracellular matrix remained intact and the extracellular matrix was able to retain its important features. This indicates that when NTIRE is used to treat a tumor adjacent to an important artery, damage to arteries within the ablation zone will be minimal, and the artery is able show critical signs of recovery within one week of electroporation. It is believed here that the ability of NTIRE to specifically target the cell membrane while preserving the extracellular matrix allows the artery to begin recovering quickly.

8.1.1.2 NTIRE for the Development of a Decellularized Tissue Scaffold

The ability of NTIRE to result in a decellularized construct 3 to 5 days after treatment led to the examination of NTIRE as a promising method for the development of a natural tissue scaffold. The versatility of applying NTIRE to the artery to develop a decellularized tissue scaffold was demonstrated by comparable results using both the plate electrodes and the endovascular electrode device. This indicates that both minimally invasive techniques and a simpler method for applying NTIRE across the artery in the lab setting can be utilized. It was shown that there is a period of time in which the artery's cells are naturally removed from the tissue before new cells repopulate the area. This decellularized arterial construct was examined as a potential tissue that could be harvested and used as an arterial graft for revascularization surgeries.

Histological analysis supported the use of NTIRE for developing this tissue scaffold. The extracellular matrix appeared undamaged, maintaining its important structural and functional components for promoting cell growth. In addition, the extracellular matrix was shown to support new cell growth within one week after treatment. Endothelial cells were seen lining the lumen. This is important not only because it indicates that the tissue has potential to regain full function, but endothelial cells are essential in preventing thrombosis from occurring. Histological analysis of the decellularized tissue also indicated that thermal damage did not occur during treatment, validating the finite element models used to chose the treatment parameters, and further demonstrating ability to predict and control NTIRE. Indeed, the ability of NTIRE to selectively target cell membranes while preserving the extracellular matrix is what gives it this unique ability to produce a decellularized scaffold *in vivo*. This method shows promise for developing a construct for use in revascularization surgeries, providing a natural scaffold that can be incorporated into the body without further complications.

8.1.2 Effect of NTIRE on the Small Intestine

The small intestine was also examined as an important tissue that could potentially limit the use of NTIRE for abdominal cancer ablation. Damage to the small intestine during localized radiation treatment as well as chemotherapy can often cause many problems and complications, even leading to discontinuance of treatment. Thus, it is important to understand how this organ responds and recovers following NTIRE in order to assess the potential use of NTIRE to treat abdominal cancers such as pancreatic cancer. In this first systemic study on the effects of NTIRE on intestinal recover, electroporation parameters were chosen from finite element modeling that would cause a strong enough electric field for irreversible electroporation while avoiding thermal damage to the tissue.

An additional finite element model of the small intestine was developed in order to investigate the effect of the heterogeneous layers of the small intestinal tissue. This model examined changes in electrical conductivity between tissue layers as well as the anisotropic effect of muscle tissue. Here, it was shown that in order to fully develop NTIRE to be used for abdominal cancers in the clinical setting, it is important to take these heterogeneous effects into account. Before that can be accomplished though, more data must be obtained for the electrical conductivities of tissues in each layer of the small intestine. In addition, experiments must be run to gain knowledge on how the electrical conductivity of the small intestinal tissue is affected by electroporation. Once this data has been obtained, it could be incorporated into a finite element model such as the one described in Chapter 6, allowing for a more accurate prediction of the electrical field distribution within the intestinal tissue and aiding in treatment planning as this technology becomes more developed for treating abdominal cancers in the clinical setting.

In order to investigate the effect of irreversible electroporation on the small intestine, the electrical parameters were applied to the rat small intestine *in vivo*. The small intestine histology was examined up to one week after treatment to gain a deeper understanding of how the molecular selectivity of NTIRE affected the small intestine's ability to regain structure. Histological analysis indicated that thermal damage to the small intestine did not occur despite obvious irreversible electroporation effects, as predicted by the finite element analysis. Complete cellular destruction to the small intestine was seen at one day after treatment, but the extracellular matrix appeared unaffected. This indicates that strong electroporation affects occurred while avoiding thermal damage. Perhaps the most promising results were that, despite complete ablation of the stem cells within the crypts in the treated area, immature epithelial cells from the boundary of the treated and untreated zone were seen migrating inward within three days after treatment, producing a new epithelial cell layer. Within one week of treatment, the tissue appeared to have regained its distinct layers, immature villi had developed within the treated area, and normal repair and replacement of the mucosa, submucosa, and muscularis was occurring. It is believed that this quick repair is due to the unique ablation mechanisms of NTIRE, selectively targeting the cell membranes, while leaving the extracellular matrix intact. Thus, important structures such as lymphatic supplies, nerves, and blood vessels were still seen to be functioning after treatment, allowing for a quick recovery. These results indicate that NTIRE may be a promising alternative for treating malignant tumors located near sensitive organs, and support the use of NTIRE for treating abdominal cancers.

8.2 FUTURE WORK

Though this work shows some exciting and promising results that could affect several different fields, it also opens up additional questions and areas that warrant further investigation. Future work based on these results can be applied to the areas of cancer treatment with NTIRE, developing NTIRE further for tissue engineering applications, and applying NTIRE to other medical treatment areas.

In order to more completely understand how NTIRE affects the tissue's ability to recover, longer recovery times could be utilized. The studies presented here examined the artery and the small intestine up to one week after treatment. The signs of recovery evident within seven days

are very promising. Nonetheless, it would be very beneficial to perform longer term studies, examining the effect of NTIRE on the recovery of critical tissues such as the artery and the small intestine for several more weeks or months, in order to gain knowledge of when complete recovery is reached and how this recovery comes about.

In addition, though the promise of using NTIRE as a method to produce a decellularized arterial scaffolds was demonstrated, a great deal of future work is necessary in order to develop this concept into an applicable methodology for scaffold development. For example, it is important to investigate the mechanical response of these decellularized arterial constructs to determine if they would be strong enough to be implanted into a host directly while maintaining structural integrity. In addition, further characterization of the decellularized artery after NTIRE treatment is important. Also, although the decellularized artery was shown to develop an endothelial layer, additional studies would be needed to examine the ability of the scaffold to be reseeded with vascular smooth muscle cells and the functional response of these arteries would have to be investigated. Implantation studies could also be utilized, examining whether or not the decellularized arterial constructs could be recellularized within a new host. Here, the concept of using NTIRE for tissue engineered scaffolds was introduced. A great deal of future work would be paramount in order to develop this idea.

Future work would also include further characterization of the electrical parameters of heterogeneous tissues such as the small intestine in order that finite element models can be developed that take into account the changes in electrical conductivity from layer to layer as well as the effect of electroporation on the tissue conductivity value. These models would be very beneficial in treatment planning and predicting the local electric fields throughout the tissue.

The results presented here for the small intestine show promise in developing NTIRE for other applications. For example, colon obstructions are often treated by stents, but this method has many potential drawbacks. NTIRE could potentially be used to ablate the colon obstruction. The small intestine results discussed in this thesis showed quick recovery of the small intestine structure, illustrating the potential ability of the colon to also recover quickly from this treatment method. Future studies would investigate how NTIRE affects the colon, potentially developing a new method to treat colon obstructions in a quick and minimally invasive fashion.

As can be seen, irreversible electroporation is a growing field. Though it has already gained a great deal of success in clinical trials for cancer treatment, there is much more work to be done to further harness this technology.

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