The Tissue Response to Infectious Burden and Implantable Devices in Healthy and Diabetic Animal Models

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2015
ABSTRACT

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Abstract

Performance of biomedical implants has been hindered by fibrosis, infection, and deficient tissue integration due in part to the body’s foreign body response. In addition, diabetes mellitus is affecting a greater number of people worldwide and in the United States. As the percentage of the population affected by diabetes increases, a larger fraction of these implanted devices will be placed in diabetic patients. Unfortunately, diabetes is often complicated by poor wound healing and a greater risk of infection, issues that could adversely affect proper acceptance of an implant. Diabetic animal models are useful in studying the response to infection as well as the tissue response to an implanted device. Therapies such as nitric oxide release have been applied to indwelling devices to mediate the foreign body response and improve the wound healing response around implants. Particularly relevant to diabetic patients are implantable glucose sensors, and so determining the diabetic tissue response to these devices is crucial to improving their lifetime and performance.

A novel outcome-based streptozotocin dosing regimen was developed to induce Type 1 diabetes in a rodent model. Male CD (Sprague-Dawley type) rats weighing 150-200 g were given three consecutive daily doses of 40 mg/kg streptozotocin (STZ) on Days 1, 2, and 3. On Day 5, tail vein blood glucose was checked. If blood glucose was not within
the target diabetic range of 350-600 mg/dl, rats received an additional dose of STZ. This procedure was repeated every 48 hours until all rats achieved target hyperglycemia. Control rats were given similar doses of vehicle (saline/citrate), which had no effect on blood glucose. After the last injection of streptozotocin, two weeks were allowed to ensure the full effects of the diabetic state would be present at device implantation. Blood glucose was measured every 2 days for diabetics and 4 days for controls for the duration of the experiment. The developed diabetic model resulted in a stable hyperglycemia for the duration of the experiment (in some cases, up to 2 months). Animals also exhibited typical symptoms of diabetes, such as minimal changes in weight, excessive thirst, and polyuria.

Infection response in the presence of implanted devices was investigated in healthy and diabetic animal models. In the healthy animal model, control stainless steel compression plates and plates coated with a nitric-oxide releasing xerogel (20% AHAP) were attached to the femurs of 12 adult rabbits. Both femurs were inoculated with $3 \times 10^6$ CFU MSSA (methicillin-sensitive Staph aureus) for a period of 20 minutes before the surgical sites were sutured. After 7 days, the wound, device, and a portion of bone were cultured. A muscle biopsy was removed and homogenized to quantify bacterial infection. Since the microbiologic data were not normally distributed, they were compared using an unpaired Wilcoxon Rank Sum Test. No significant differences in
bacterial burden were observed between the control and NO-eluting devices, however the study did find a high correlation of temperature of the adjacent muscle at implantation with the ensuing bacterial content. In the diabetic animal model, a novel dosing regimen of streptozotocin based on a target blood glucose of 350-600 mg/dl was used to induce type-1 diabetes. Stable hyperglycemia was maintained for 21 days. Two weeks after achieving the target hyperglycemia, stainless steel fracture plates were secured to each femur with stainless steel screws approximately 3 mm in length. The implant site on the right side of the animal was inoculated with $3 \times 10^7$ CFU of methicillin-sensitive S. aureus while the left side served as a control. After seven days, quantitative bacterial count was performed at explantation and no cross-over of bacteria was detected from the inoculated side to the non-inoculated side. Infection after S aureus inoculation in the presence of an implanted device was significantly higher in diabetic animals when compared to that of control animals (p = 0.0003, Wilcoxin Rank-Sum Test) supporting the hypothesis that diabetes adversely affects the ability to fight infection in the presence of an indwelling device in an animal model. There was not a significant difference detected in the infectious burden for the non-inoculated limb (left) between the diabetic and non-diabetic groups when compared using a Wilcoxon Rank-Sum Test (p = 0.0682), however this near-significance suggests that even in the absence of an introduced inoculum, diabetes increases infection susceptibility in the presence of an implant.
Nitric oxide (NO) release can be used to mediate the foreign body response around implanted devices. NO also has antibacterial properties that may enhance the body’s immune response to implant-associated infection. Additionally, diabetic wounds are characterized by nitric oxide deficiency, and thus NO supplementation may promote better wound healing and implant acceptance in diabetics. The use of nitric oxide to modulate the tissue response to indwelling implants was explored in two studies. In vivo glucose recovery of subcutaneously implanted NO-releasing microdialysis probes was evaluated in a healthy rat model using saturated NO solutions that provided a steady release of NO. A constant NO flux of 162 pmol cm$^{-2}$ s$^{-1}$ was perfused through the probe membrane for 8 hours daily. The in vivo effects of increased localized NO were evaluated by monitoring glucose recovery over a 14-day period. Beginning at 7 days, significant differences in glucose recovery between the control and NO-releasing probes were observed. At the 14-day time point, histological analysis revealed decreased inflammatory cell density at the probe surface and a thinner collagen capsule. In the second study, polyurethane-coated wires with varying NO release properties were implanted subcutaneously in 17 Yorkshire piglets with time points of 3, 7, 21, and 42 days. To create the NO-releasing coating, the NO-releasing vehicle (i.e., PROLI/NO, AEAP3 or MPTMS nanoparticles) was dispersed into EtOH (2.5 mL) at concentrations of 36 or 72 mg/mL. This solution was then mixed with an equal volume of 50:50 wt%
HPU/TPU (160 mg/mL total PU). Effects of NO release were analyzed using histological data. These data were analyzed using a non-parametric Wilcoxon rank-sum test.

Coatings with short NO release durations (i.e., 24 h) failed to reduce collagen capsule thickness at 3 and 6 weeks. Longer release durations (3 and 14d) however significantly reduced collagen capsule thickness at longer timepoints. The acute inflammatory response was significantly affected by coatings with the longest duration and greatest dose of NO release. However these benefits were not realized at later timepoints, suggesting that NO must be actively released in order to influence inflammatory response.

The tissue response to percutaneously implanted glucose sensors was investigated in healthy and diabetic rats. A multi-dose regimen of streptozotocin was used to induce diabetes in experimental rats. Three types of functional, implantable glucose sensors, supplied by Medtronic® were used: Sof™ sensor, Enlite™ sensor, and Enlite 2™ sensor. The sensors were percutaneously implanted in the rat dorsum subcutaneous space. MiniLink™ transmitters were attached to the rats, permitting continuous glucose monitoring. At 3 days, 1 week, and 4 weeks, tissue directly adjacent to the sensors was evaluated for collagen encapsulation, density of any collagen encapsulation, inflammatory response as measured via inflammatory cell density, and microvessel density. These endpoints were evaluated histologically via Masson’s trichrome,
Hoechst, H&E, and CD31 staining. Additionally, continuous functional sensor data was evaluated for sensor accuracy, attenuation, and lag time. Histological analyses revealed few significant differences in collagen thickness among different sensors, in different tissue types, or over time. In general, Masson’s trichrome-stained images seem to suggest a balance between collagen capsule formation and inflammatory cell density. As inflammation increased adjacent to sensors over time, collagen capsule thickness decreased somewhat and stabilized. Collagen capsule formation was most evident adjacent to the plastic tubing portion of the sensor whereas inflammation was greatest adjacent to the sensing electrode. Likewise, few significant differences in collagen density index (CDI) were observed among sensor types, tissue types, or over time. CDI remained relatively constant over time for all sensors. Analysis of inflammatory cell density in general revealed a greater inflammatory response adjacent to percutaneous Enlite sensors, though these results were not significant. Additionally, inflammatory cell density was generally greater adjacent to non-diabetic sensors, however this result was also not significant. Inflammatory cell density increased or remained stable over time for all sensor types, suggesting that the presence of percutaneously-implanted sensors produces a chronic inflammatory response that does not resolve. Vascularity adjacent to implanted sensors remained generally stable over time, sometimes decreasing but not significantly. At the 1-month timepoint, no significant differences in vasculature were seen among sensor types. A balance also appears to exist for
microvessel and inflammatory cell densities. The non-diabetic percutaneous Enlite sensor had the greatest microvessel density at earlier timepoints, while also having the greatest inflammatory cell density. However, at later timepoints, microvessel density decreased somewhat as inflammation increased somewhat. Finally, analysis of sensor performance showed significant sensor failure at longer timepoints. Sensitivity decreased somewhat for all sensors except for the non-diabetic Enlite sensor, which in general had greater overall sensitivity in comparison to the non-diabetic Sof sensor. Lag time was relatively similar among all sensor types, tissue types, and over time. MARD values were considerably lower for diabetic sensors for the Day 1 bolus, but were generally similar for all sensors at the 1-week bolus. These results suggest that the diabetic foreign body response, while somewhat decreased, is not significantly different than that in non-diabetic tissue. In addition, the design of the Enlite™ and Enlite 2™ sensors promoted a more aggressive inflammatory response despite being smaller and more flexible in design. Most evident from the results was the presence of a chronic inflammatory response adjacent to the percutaneously-implanted sensors, which likely contributed to the high rate of sensor failure over time.
Dedication

I dedicate all this hard work to my husband.
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Part I: BACKGROUND

Chapter 1: Purpose and Specific Aims

1.1 Purpose

The purpose of this dissertation was to understand the foreign body response to implantable materials in control and diabetic animals as well as to address some of the challenges associated with indwelling devices, such as infection and micromotion. These findings can in turn be used to guide implantable glucose sensor design to optimize sensor function and performance.

1.2 Specific Aims

1.2.1 Establishment of a diabetic animal model

A novel dosing regimen based on target blood glucose outcomes was used to induce Type 1 diabetes in a rat model. Rats received variable (at least 3) doses of streptozotocin at 40 mg/kg until they achieved target blood glucose levels of 350-600 mg/dl. Blood glucose levels were measured using standard OneTouch Ultra or Aviva AccuCheck glucometers. Using this method, we have been able to reliably induce diabetes in 100% of our wild-type rats.
1.2.2: **Investigation of infection response in healthy and diabetic animals**

Orthopedic implant animal models were used to investigate infection response in two separate studies. Control stainless steel compression plates and plates coated with a nitric-oxide releasing xerogel were attached to the femurs of healthy adult rabbits. After bilateral inoculation with *S. aureus*, quantitative microbiology was performed to assess antibacterial efficacy of the nitric oxide-releasing coatings after 7 days. These same stainless steel plates were attached to the femurs of healthy and diabetic CD rats. After inoculation of one limb, bacterial burden was quantified after 7 days to compare the immune response in healthy and diabetic animals.

1.2.3: **The use of nitric oxide to modulate the tissue response to indwelling implants**

The use of nitric oxide to modulate the tissue response to indwelling implants was explored in two studies. *In vivo* glucose recovery of subcutaneously implanted NO-releasing microdialysis probes was evaluated in a healthy rat model using saturated NO solutions that provided a steady release of NO. A constant NO flux of 162 pmol cm$^{-2}$ s$^{-1}$ was perfused through the probe membrane for 8 hours daily. The *in vivo* effects of increased localized NO were evaluated by monitoring glucose recovery over a 14-day period. In the second study, polyurethane-coated wires with varying NO release properties were implanted subcutaneously in 17 Yorkshire piglets with time points of 3, 7, 21, and 42 days. For both studies, effects of NO release were analyzed using
histological data measuring collagen capsule formation, collagen density, and inflammatory cell density.

1.2.4: The tissue response to functional glucose sensors in diabetic and non-diabetic animals

Functional glucose sensors were percutaneously implanted in healthy and diabetic rats for 3 days, 1 week, and 1 month. Interstitial glucose concentrations were continuously monitored in rats throughout the experiment, with insulin and glucose bolus challenges performed 24 hours post sensor implantation and once a week for longer timepoints. Total microvessel count and inflammatory cell density within 200µm of the sensor surfaces were evaluated with histology. Collagen capsule formation and collagen density were also analyzed.
Chapter 2: The molecular pathogenesis of wound healing in diabetic patients


2.1: Overview

Diabetes is becoming increasingly prevalent worldwide. In 2007, the American Diabetes Association estimated nearly 25 million were affected by diabetes in the United States alone\(^1\). One of the most common complications of diabetes is an altered or deficient healing of wounds\(^2\). Currently, over 100 physiologic factors have been discovered that contribute to wound healing deficiencies in diabetics\(^3\). These include impaired growth factor production\(^4^6\), angiogenic response\(^6^7\), macrophage function\(^8\), collagen accumulation, quantity of granulation tissue\(^6\), fibroblast migration and proliferation, number of epidermal nerves\(^9\), and balance between ECM component accumulation and remodeling by MMPs\(^10\). In normal tissues, wound healing is characterized by efficient inflammatory cell recruitment in response to a variety of chemokines, including MIP-2 and MCP-1. Chemokine and cytokine signaling stimulate the production of growth factors that promote matrix formation, angiogenesis, and re-epithelialization. In diabetic wounds however, decreased chemokine expression results in decreased growth factor production and delayed inflammatory cell infiltration\(^11\). Alterations in many chemokines and growth factors in diabetic wounds lead to impairments in angiogenesis,
matrix formation, and re-epithelialization (Table 1), deficiencies that are detrimental not only to cutaneous wounds, but to internal wounds adjacent to surgical implants\textsuperscript{11-12}.

During later stages of diabetic wound healing, persistence of inflammatory cells within injured tissue results in continued damage and turnover due to increased expression of IL-1β, TNF-α, and MMPs\textsuperscript{12}.

<table>
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<th>Cytokine and growth factors</th>
<th>Normal role in wound healing</th>
<th>Expression in diabetic wound healing</th>
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| IGF-1                       | Promotion of reepithelialisation  
Keratinocyte and fibroblast proliferation  
Endothelial cell activation | Decreased |
| TFG-β1                      | Chemoattractant (keratinocytes, fibroblasts, inflammatory cells)  
ECM deposition  
Promotes angiogenesis | Decreased |
| PDGF                        | Fibroblast activation  
Promotes angiogenesis  
ECM deposition  
MMP synthesis | Decreased |
| EGF                         | ECM deposition  
Keratinocyte migration and Proliferation | Decreased |
| IL-8                        | Keratinocyte proliferation  
Macrophage chemotaxis  
Neutrophil chemotaxis | Decreased |
| Angiopoietin-2              | Disrupts blood vessel formation | Increased |

EGF, endothelial growth factor; IGF-1, insulin-like growth factor-1; IL-8, interleukin-8; MMP, matrix metalloprotease; PDGF, platelet-derived growth factor; TFG-β1, transforming growth factor-β1.

Other aspects of diabetic wound healing include reduced cell proliferation in the wound tissue, slowed onset of myofibroblast differentiation, and increased levels of apoptosis during inappropriate stages of the healing process\textsuperscript{13}. Keratinocytes show an absence of
migration, hyperproliferation, and incomplete differentiation while fibroblasts exhibit decreased migration and proliferation.

Some of these factors are particularly of significance in the tissue integration of an indwelling medical device. Decreased amounts of VEGF and other growth factors influence the development of blood vessels crucial to proper wound healing; poor angiogenesis results in poor implant integration with surrounding tissue\textsuperscript{14-16}. Decreased capillary concentration around an implant results in poorly perfused tissue that is inconsistent with healthy tissue. Additionally, diabetic wound sites were found to be stiffer due to greater collagen accumulation and cross-linking, and decreased amounts of growth factors such as PDGF and EGF result in poor matrix formation\textsuperscript{12, 14}. Finally, NO, an important mediator in wound healing, is deficient in diabetic wounds\textsuperscript{17}. In addition to its role in wound healing, nitric oxide has antibacterial properties that can partly assist in combating implant-associated infections\textsuperscript{18}.

\textbf{2.2: Mechanisms of wound healing in healthy people vs. people with diabetes}

Wound healing is a complex cellular response to injury and involves the activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. These cells release growth factors, cytokines, and chemokines that coordinate and maintain healing in healthy individuals. Shortly after injury, hypoxia is induced and VEGF released by macrophages, fibroblasts, and epithelial cells initiates the phosphorylation and
activation of eNOS in the bone marrow. Rising NO levels initiate the mobilization of bone marrow EPCs to the circulation. Chemokines direct these EPCs to the wound site where they participate in vasculogenesis.

According to a murine model of diabetes, eNOS phosphorylation in the bone marrow is impaired as evidenced by significantly limited EPC migration from the bone marrow into the circulation. In addition to deficient activation of eNOS, expression of key chemokines is decreased in epithelial cells and myofibroblasts of the diabetic model. EPCs do not receive the signals necessary to relocate to the wound site, and healing is impaired due to decreased angiogenesis.

2.3: The time-course of healing

2.3.1: The course of normal wound healing

Much of what is known about diabetic wound healing has been derived from experimental wounds in animals. The biological and molecular events occurring after cutaneous injury are generally divided into four phases of repair: coagulation, inflammation, migration-proliferation and matrix deposition, and remodeling (Figure 1, Table 2). The phases overlap considerably, but healthy wounds exhibit linear progression. Conversely, chronic non-healing wounds such as those often seen in diabetics have a disorganized progression of wound healing, with some wound areas present in different phases simultaneously.
During coagulation, the first phase of wound healing, a fibrin plug forms shortly after injury and inflammatory cells quickly migrate to the wound. Platelets within the plug release growth factors such as PGDF and TGF-β that are responsible for cell recruitment. Though hypoxia results from damage to blood vessels, beneficial effects include increased keratinocyte migration, early angiogenesis, proliferation and expansion of fibroblasts, and transcription and synthesis of necessary growth factors and cytokines. These processes occur within hours of injury.

During inflammation, which takes place within about 2-3 days after injury, neutrophils and monocytes arrive and assist in wound debridement and release growth factors. Several other inflammatory and dermal cells follow including macrophages, fibroblasts, and endothelial cells. Granulation tissue begins to form.

Occurring over several days after injury, the migration-proliferation phase is marked by epidermal resurfacing, fibroplasia, angiogenesis, ECM deposition, and initial wound contraction. Contraction is promoted by the formation and organization of granulation tissue and the presence of myofibroblasts. Angiogenesis supplies the wound area with oxygen and nutrients. In the weeks and months after injury, a scar is formed at the wound site that is remodeled over time. ECM degrades and further contraction occurs6,19.
Figure 1: The time course of the different cells appearing in the wound during the healing process. Macrophages and neutrophils are predominant during inflammation, whereas lymphocytes peak somewhat later and fibroblasts are predominant during the proliferative phase.
Table 2: Phases of wound healing, major types of cells involved in each phase, and selected specific events.

<table>
<thead>
<tr>
<th>Phases</th>
<th>Main cell types</th>
<th>Specific events</th>
<th>Timeframe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulation</td>
<td>Platelets, Neutrophils, monocytes</td>
<td>Platelet aggregation and release of fibronogen fragments and other proinflammatory mediators</td>
<td>Within hours</td>
</tr>
<tr>
<td></td>
<td>Fibrin plug formation, release of growth factors, cytokines, hypoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>Macrophages</td>
<td>Selectins slow down blood cells and binding to integrins -&gt; diapedesis</td>
<td>Within days</td>
</tr>
<tr>
<td></td>
<td>Cell recruitment and chemotaxis, wound debridement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migration/Proliferation</td>
<td>Keratinocytes, fibroblasts, endothelial cells</td>
<td>Hemidesmosome breakdown -&gt; keratinocyte migration Cross-talk between MMPs, integrins, cells -&gt; cell migration, ECM production</td>
<td>Within weeks to months</td>
</tr>
<tr>
<td></td>
<td>Epidermal resurfacing, fibroplasia, angiogenesis, ECM deposition, contraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remodeling</td>
<td>Myofibroblasts</td>
<td>Phenotypic switch to myofibroblasts from fibroblasts</td>
<td>Within weeks to months</td>
</tr>
<tr>
<td></td>
<td>Scar formation and revision, ECM degradation, further contraction and tensile strength</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3.2: The effect of diabetes on wound healing

In diabetics, all stages of the wound healing cascade are affected. Many studies have shown a decreased inflammatory response in diabetic wounds, including decreased chemotaxis, bacterial killing and phagocytosis, and antioxidant levels resulting from the impaired function of neutrophils and macrophages. During later stages of healing, diabetic wounds show growth factor depletion, raised glucocorticoid concentrations, diminished cell proliferation, and increased apoptosis. Several days after injury, neovascularization is decreased and granulation tissue is poorly developed. The alterations in cell recruitment and proliferation seen in diabetes may complicate integration of implanted devices; decreased granulation tissue formation
likely results in an unstable environment for a device. Additionally, poor blood vessel
development and perfusion may result in the formation of an avascular tissue barrier
around a device when optimally the area around an implant should be identical to
uninjured tissue. In particular, decreased neovascularization is a considerable
impediment to the function of glucose sensors, as distance from the sensor to glucose in
the bloodstream can affect the accuracy of glucose measurements.
Chapter 3:  Diabetes and biomedical implants

With the increasing prevalence of diabetes, a considerable number of diabetic patients will require some sort of indwelling medical device in their lifetime as the use of devices such as orthopedic implants, breast implants, and glucose sensors is rapidly expanding. In 2004, 600,000 joint prostheses and 2,000,000 fracture-fixation devices were implanted in the United States. Breast implants accounted for 130,000 implanted medical devices, while statistics estimated that nearly 800,000 dental implants were done in the United States in 2004 (Table 3). As the percentage of the population affected by diabetes increases, a larger fraction of these implanted devices will be placed in diabetic patients. Specifically, percutaneous glucose sensors are designed for use in diabetic patients. Medtronic, Inc., which manufactures the MiniMed glucose sensor, has sold over one million sensors as of 2008, which have been implanted in patients worldwide.

Performance of biomedical implants has been hindered by fibrosis, infections, and deficient tissue integration due in part to the body’s foreign body response. Additionally, micromotion of the implant itself may induce a more aggressive foreign body reaction, further impairing implant function and integration. Implant-associated infections account for half of the nearly 2 million nosocomial infections in the United States yearly. While most of these infections involve catheters, infections involving surgical implants are generally more difficult to manage because they usually require a longer period of antibiotic therapy and repeated surgical procedures. Concerning
glucose sensors, while there is no reported research on the infection rates of such
devices, infection is a potential concern due to the tract available for bacterial migration.
Some groups have engineered coatings for glucose sensors to prevent or mediate
bacterial infection. The Schoenfisch group has coated glucose sensors with nitric-oxide
releasing xerogels, which have antibacterial properties$^{35}$. In addition, device function
and performance can be adversely affected if the foreign body response elicited by the
device culminates in fibrotic encapsulation.
The common complications of diabetes, altered healing of wounds and a higher susceptibility to infections, can likely complicate the body’s acceptance of an implant and lead to greater rates of rejection. While some research has investigated the effect of diabetes on the body’s healing ability, most particularly the case of diabetic foot ulcers, these findings have not been applied to understanding a diabetic’s response to an


<table>
<thead>
<tr>
<th>Implant</th>
<th>Implants Inserted in the U.S. Annually</th>
<th>Projected Infections of Implants Annually</th>
<th>Average Rate of Infection</th>
<th>Preferred Practice of Surgical Replacement</th>
<th>Estimated Average Cost of Combined Medical and Surgical Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical heart valve</td>
<td>85,000</td>
<td>3,400</td>
<td>4</td>
<td>1</td>
<td>50,000</td>
</tr>
<tr>
<td>Vascular graft</td>
<td>450,000</td>
<td>16,000</td>
<td>4</td>
<td>1 or 2</td>
<td>40,000</td>
</tr>
<tr>
<td>Pacemaker–defibrillator</td>
<td>300,000</td>
<td>12,000</td>
<td>4</td>
<td>2</td>
<td>35,000</td>
</tr>
<tr>
<td>Ventricular assist device</td>
<td>700</td>
<td>280</td>
<td>40</td>
<td>1</td>
<td>50,000</td>
</tr>
<tr>
<td>Orthopedic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Joint prosthesis</td>
<td>600,000</td>
<td>12,000</td>
<td>2</td>
<td>2</td>
<td>30,000</td>
</tr>
<tr>
<td>Fracture-fixation device</td>
<td>2,000,000</td>
<td>100,000</td>
<td>5</td>
<td>1 or 2</td>
<td>15,000</td>
</tr>
<tr>
<td>Neurosurgical — ventricular shunt</td>
<td>40,000</td>
<td>2,400</td>
<td>6</td>
<td>2</td>
<td>50,000</td>
</tr>
<tr>
<td>Plastic — mammary implant (pair)</td>
<td>130,000</td>
<td>2,600</td>
<td>2</td>
<td>2</td>
<td>20,000</td>
</tr>
<tr>
<td>Urologic — inflatable penile implant</td>
<td>15,000</td>
<td>450</td>
<td>3</td>
<td>2</td>
<td>35,000</td>
</tr>
</tbody>
</table>

* The information is from published studies, market reports, and data provided by medical and surgical organizations, physicians, and device-manufacturing companies. The average costs reflect the usual charges by private institutions [taking into consideration that portions of the antibiotic courses, particularly prolonged courses, are sometimes administered in an outpatient setting] and exclude loss of income because of infection.

† The average rate of infection refers to initially inserted implants, which are less likely to become infected than replacement implants. For mechanical heart valves, the average rate refers to the incidence of prosthetic-valve endocarditis within 60 months after implantation. For ventricular assist devices, it refers to infections documented within three months after implantation, and for ventricular shunts, it refers to infections in adults and children, even though children are more likely to become infected.

‡ The average rate of infection of vascular grafts refers to arteriovenous, femoropopliteal, and aortic grafts combined.

§ The average cost of treatment refers to infections associated with all three types of vascular grafts.

∥ The average cost of treatment represents a weighted average of the costs of treating infections of pacemakers ($25,000) and pacemaker–defibrillator systems ($50,000); the difference in the cost of treating infections of these two systems is largely attributed to the difference in the average cost to a hospital of a pacemaker ($5,000) and a pacemaker–defibrillator system ($30,000).

¶ Fracture-fixation devices include intramedullary nails, external-fixation pins (which are more likely to become infected than intramedullary nails), plates, and screws. A one-stage procedure is usually performed in patients with bone union, and a two-stage procedure in the absence of bone union. The average cost of treatment refers to infections associated with the various types of fracture-fixation devices. Treatment of infections of intramedullary nails is more expensive than treatment of infections of external-fixation pins (average costs, $25,000 vs. $5,000).
indwelling implant. Little research has focused on how diabetes alters the internal wound healing around an implant. In normal tissues, the presence of an implant can lead to the foreign body reaction and eventual fibrotic encapsulation, which can severely limit the device’s performance. For example, fibrotic encapsulation of glucose sensors prevents accurate measurements of blood glucose levels because glucose must diffuse through an abnormal tissue barrier before its concentration can be read by the sensor. The ensuing lag time results in different concentrations in the blood and at the sensor surface. It is unknown if a similar foreign body response occurs in diabetics.

Considerable research has focused on dental and orthopedic implants in diabetics, however these implants are atypical of soft tissue implants. In regards to soft tissue implants, subcutaneous and percutaneous devices of varying materials have been implanted in diabetic animal models to study the wound healing response – not necessarily to the implanted device. Additionally, implants releasing therapeutic agents such as NO have been reported\textsuperscript{17}, but these studies emphasize techniques to enhance wound healing in diabetics. Therefore, it is crucial to study implant healing in diabetics because understanding how the foreign body reaction is regulated in diabetics will allow for the development of new therapies to reduce fibrosis and increase the utility of implanted devices.
3.1: Dental and orthopedic implants

Several experimental models have investigated the effect of diabetes on the osseointegration of dental and orthopedic implants. Periodontal disease is a frequent complication of diabetes. The failure rate of bone implants is higher in diabetic patients, particularly if the disease is poorly controlled. Studies have shown that diabetes induces an alteration in the bone remodeling processes. Deficient mineralization then results in decreased osseointegration of the implant. Reduction in contact between the bone and the implant is present despite comparable bone formation in diabetic and control animal models and infection of oral implants is a continual danger. Antibiotics are often recommended for diabetics due to their greater susceptibility for infection.

In the orthopedic literature, research examining differential healing in diabetic animal models has focused primarily on bone fractures since clinically, diabetes has been shown to alter bone composition, reduce bone mass, and impair fracture healing in humans. The mechanism of delayed bone healing in diabetic animal models has yet to be fully elucidated, but impaired osteoblast and osteoclast function, as well as altered chondrogenesis have been proposed.

3.2: Subcutaneous implants

One recent study investigated wound healing in a diabetic baboon model using a polystyrene implant. Drum devices were implanted subcutaneously in the thighs of
long-term diabetic baboons. Implants were removed after 2 and 4 weeks and analyzed for granulation tissue and inflammatory cell migration into the drums. Granulation tissue was reduced at both time points in diabetic baboons and vessel lumen areas were greater at 4 weeks compared to control animals. Fewer macrophages were present in diabetic tissue while neutrophils were prominent. Lastly, after 4 weeks, diabetic wound tissue exhibited less connective tissue ingrowth, resulting in a coarser, more disorganized tissue structure. There was no mention of fibrotic capsule formation.

3.3: Percutaneous implants

Gerritsen et al. investigated the soft tissue response to subcutaneous and percutaneous devices composed of titanium fiber mesh in diabetic and non-diabetic rabbits. The percutaneous device consisted of a subcutaneous component of titanium fibers and a percutaneous segment that was attached via threaded hole. Titanium mesh was also implanted subcutaneously to examine the difference in tissue response to subcutaneous and percutaneous implants. The group found a greater number of infectious complications around percutaneous implants in severely diabetic animals. Severe diabetes adversely affected matrix maturation and delayed neovascularization in both implant types. Inflammatory cells were present in higher numbers around percutaneous devices in severely diabetic rabbits; a nearly 40% increase in inflammatory cells was seen around these devices when compared to the subcutaneous devices.
Chapter 4: Diabetes and infection

Diabetics are generally more susceptible to infections, which presents a significant obstacle to implant integration. Infections result in considerable morbidity and mortality in diabetic patients even in the absence of an indwelling medical device. In a study conducted by Bertoni et al, 9,208 adults aged 30-74 years in 1976-1980 were followed over a period of 12-16 years. Thirty-six infection-related deaths occurred among 533 diabetic adults versus 265 deaths in 8,675 adults without diabetes (4.7 vs. 1.5 per 1,000 person-years, P < 0.001), suggesting that diabetic adults are at greater risk for infection-related mortality. In the presence of an implant, several large-scale retrospective studies have found diabetic patients have increased rates of surgical site infections when compared to healthy individuals. In diabetics undergoing spondylolithesis, the infection rate was 10.3% compared to 0.7% in non-diabetic patients. Extensive research has been done to elicit the differences in infection response in diabetic and non-diabetic patients, with most studies suggesting defects in cellular innate immunity as being primarily responsible for the decreased ability to fight infection. Additionally, some studies have shown certain microorganisms exhibit an increased adherence to diabetic cells. Understanding the diabetic response to infection can be useful in improving implanted device longevity.

4.1: The normal immune response to infection

The normal immune response in wound healing can be divided into the innate and the
adaptive immune response. The repeated observations on previous publications suggest that only the innate response is adversely altered in diabetic foreign body reaction.

In the innate response, cells such as T cells, natural killer cells, neutrophils, mast cells and macrophages immediately recognize an antigen and attack the carrier in a non-specific manner. The response is short-lived and does not confer lasting, protective immunity to the host\textsuperscript{50}. Innate immunity provides immediate defense against infection by recruiting immune cells via cytokine signaling, assisting in the activation of the complement cascade, identifying and removing foreign substances, and activating the adaptive immune system through antigen presentation. Many studies have implicated the following constituent factors of the innate immune system as being most responsible for the alterations in diabetic healing: defects in PMNLs, altered monocytes and mast macrophages, and increased adherence of microorganisms to diabetic cells.

The adaptive immune system consists of specialized systemic cells and processes that eliminate or prevent pathogenic challenges. The adaptive immune system is activated by the innate immune system and provides the ability to recognize and remember specific pathogens. Future infection by those pathogens will result in a stronger immune response. The humoral aspect of the adaptive immune system is mediated by
secreted antibodies produced by B cells. Antibodies bind to antigen markers on the surfaces of invading microbes such as viruses or bacteria, which flags them for destruction.

The adaptive humoral immune response is the main defense against invasion of extracellular bacteria. Production of antibodies assists in the removal of bacteria and the inactivation of bacterial toxins. Antibodies that bind to available antigens on the surface of bacteria assist in activating the complement cascade, resulting in increased phagocytosis, clearance, and the localized production of immune effector molecules that assist in the inflammatory response. Effector molecules such as C3a and C5a function as anaphylatoxins to promote local mast-cell degranulation, vasodilation, and extravasation of lymphocytes and neutrophils from the blood into the tissue. Other complement products act as chemotactic factors for neutrophils and macrophages, increasing the number of phagocytic cells at the infection site.

4.2: Immune deficiencies in a diabetic patient

Deficiencies in diabetic patients appear to occur primarily within the innate immune system. For instance, adaptive humoral immunity in diabetics expresses normal levels of serum antibodies and normal response to vaccinations\textsuperscript{48,51-53}. In adaptive cellular immunity, the proliferative response of lymphocytes in diabetics is inhibited with some stimuli, such as \textit{Staphylococcus aureus} and phytohemagglutinin, and normal with
4.2.1: Defects in cellular innate immunity

**Cellular innate immunity – defects in PMNLs**

Previous studies involving PMNLs in diabetics have reported abnormalities in the adherence, chemotaxis, phagocytosis, oxidative properties, and intracellular killing of these cells. Reportedly, PMNL chemotaxis is significantly lower in diabetic patients even after stimulation when compared to controls. In addition, PMNL phagocytotic and killing capacity has been found to be lower in diabetics (Table 3), leading to a poorer ability to fight off infection. The pathogenesis of these abnormalities is not entirely known, and conflicting studies have emerged. It also seems that proper glucose control can rectify these immune deficiencies so the true influence of PMNL defects on infection susceptibility is still uncertain.

**Cellular innate immunity – defects in monocytes/macrophages**

Diabetic monocytes exhibit both impaired chemotaxis and phagocytosis most likely resulting from an intrinsic monocyte defect. One study showed that children with Type 1 diabetes had a lower immune response to intradermal administration of hepatitis B vaccine when compared with control children. In this case, defective macrophage function appeared to be responsible.
Table 4: Summary of the different immune dysfunctions found in diabetic patients (reprinted from Geerlings and Hoepelman, *FEMS Immunology and Medical Microbiology* 1999; 26:259-265).

<table>
<thead>
<tr>
<th>Immune Dysfunctions</th>
<th>Humoral</th>
<th>Cellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement</td>
<td>↓</td>
<td>PMNs</td>
</tr>
<tr>
<td>Cytokines without stimulation</td>
<td>↑</td>
<td>Monocytes/macrophages</td>
</tr>
<tr>
<td>Cytokines after stimulation</td>
<td>↑ =</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>↑ =</td>
<td></td>
</tr>
</tbody>
</table>

\(\downarrow\) means that this function is decreased, \(\uparrow\) means that this function is increased in diabetic patients compared with nondiabetic controls.

4.2.2: Adherence of microorganisms to diabetic cells

Microorganism adherence to mucosal or epithelial cells is an early step in the pathogenesis of infections. *C. albicans* is an infectious microorganism commonly found in diabetic patients. Researchers determined several risk factors that could increase the risk of contracting the infection in diabetics, including a lower age, higher HbA1c level, the presence of glucosuria, and cigarette smoking. It is theorized that poor regulation of high blood glucose levels and other factors lead to altered receptors on the patient’s cells that exhibit greater adherence for infectious organisms.

4.3: Immune dysfunction and infection response in diabetic animal models

Animal models have played a key role in the attempt to elucidate the mechanism of immune dysfunction in diabetics. Although these findings are not broadly applicable, if taken into proper context they can provide useful insight into the underlying physiology. Leukocyte dysfunction in diabetic animals has been studied extensively, and the decreased inflammatory response observed in diabetic rodents has been
attributed to many factors including a decreased microvascular response to histamine and bradykinin, reduced mast cell degranulation, and reduced levels of TNF-alpha and IL-1\textsuperscript{55}. Additionally, diabetic rodents infected with S. aureus have shown altered neutrophil chemotaxis and a decreased production of reactive oxygen species\textsuperscript{56}. Macrophage dysfunction in STZ-induced diabetic rats has also been studied, showing that mice with prolonged uncontrolled diabetes have decreased cytokine production, antigen presentation, and phagocytosis\textsuperscript{57}. 
Chapter 5: Enhancing wound healing in diabetes: experimental therapies

Many therapies have been developed to address the deficiencies present in the diabetic wound. Most experimental healing therapies have been applied to skin incisions of varying thickness; previous studies have investigated healing of these incisions in a variety of animal models from mice to baboons\textsuperscript{14, 59}. Other studies involve subcutaneous implants that can release healing promoters, such as NO\textsuperscript{17}. These various treatments have shown promise in some animal models, but not all have been attempted in human patients.

5.1: Nitric oxide supplementation

Diabetic wounds are characterized by NO deficiency. One group\textsuperscript{17} developed a method for delivering NO donor to the wound site. Polyvinyl alcohol sponges were implanted subcutaneously through dorsal skin incisions created in diabetic and non-diabetic rats. Half of the implants were treated with the NO donor molsidomine. The study found that wound breaking strength and MMP-2 activity were significantly increased by exogenous NO. NO treatment however had no effect on the delayed inflammatory reaction in diabetes. The results suggested NO therapy can partially enhance the wound healing response in a diabetic animal model.

5.2: Growth factor therapy

Concentrations of many growth factors are decreased in diabetic wounds. In one model\textsuperscript{58}, full-thickness skin wounds were made in the backs of genetically diabetic mice.
These wounds were characterized by delayed infiltration of inflammatory cells, poor granulation tissue formation, and delayed wound closure when compared to wounds in non-diabetic littermates. Diabetic wounds were treated with recombinant human PDGF and basic FGF individually and in combination. Treatment with the growth factors resulted in increased fibroblasts and capillaries at the wound site and significantly greater wound closure after 21 days.

Enhancing VEGF production in a diabetic wound can promote angiogenesis. One group used Simvastatin, a drug typically used to reduce cholesterol, to stimulate angiogenesis in a diabetic mouse model. Simvastatin was administered daily to diabetic and non-diabetic mice with incisional skin wounds. Treatment with Simvastatin increased VEGF mRNA and protein expression in diabetic mice and enhanced NO production, successfully restoring wound healing capabilities.
Chapter 6: Animal models of diabetes mellitus

Many models exist that accurately portray aspects of both Type 1 and Type 2 diabetes. Type 1 diabetes mellitus results from the specific autoimmune destruction of the insulin-producing pancreatic beta cells. Hyperglycemia then results due to the lack or reduced amount of insulin. Type 2 diabetes encompasses a heterogeneous group of disorders characterized by insulin resistance and impaired insulin secretion. A number of toxins can induce Type 1 diabetes by damaging pancreatic beta cells. Additionally, selective inbreeding has resulted in strains that are fairly reasonable models of the disease states of Type 1 and 2 diabetes, and even obesity and insulin resistance. Animal models have been instrumental in the study of the pathogenesis of diabetes and its complications as well as the testing of new diabetic treatments before they can be considered for clinical use\textsuperscript{60}.

6.1: Animal models of Type 1 diabetes mellitus

Type 1 diabetes mellitus results from the specific autoimmune destruction of the insulin-producing pancreatic beta cells. Subsequently, hyperglycemia results due to the lack or reduced amount of insulin.

6.1.1: Surgically and chemically induced Type 1 diabetic animal models

The effects of hyperglycemia can be studied most simply in animals by partial or complete removal of the pancreas. Hyperglycemia can also be induced non-surgically by administering toxins, such as streptozotocin and alloxan, that damage the pancreas\textsuperscript{61}.
Streptozotocin is a broad-spectrum antibiotic that has alkylating properties, and thus modifies biological macromolecules, fragments DNA, and destroys the beta cells causing a state of insulin-dependent diabetes. The drug can be administered intravenously or intraperitoneally in a single, large dose or smaller, repeated doses over a period of days. A single large dose (60mg/kg) of streptozotocin is sufficient to produce diabetes in rodents, though repeated smaller doses are equally effective and in fact, may more reliably produce consistent models. Both surgical and chemically-induced pancreatic damage are useful in investigating what complications arise due to hyperglycemia. For example, the multiple low-dose streptozotocin model has been used to investigate the immunological pathways that lead to insulitis and β cell death, and both methods performed on female animals are helpful in studying the effect of gestational diabetes on offspring.

6.1.2: Spontaneous animal models of Type 1 diabetes

Certain animal strains spontaneously develop diseases that are similar to Type 1 diabetes. These models are produced by selectively inbreeding for generations based on hyperglycemia. Inbreeding results in the enrichment of a variety of genes and phenotypes, which may differ in relevancy to the pathophysiology of diabetes in animals or humans. Two of the most common spontaneous models for Type 1 diabetes are the NOD mouse and the BB rat because they spontaneously develop diabetes similarly to humans. Nevertheless, new animal models are continuously being
developed because of the inability of a single model to completely represent the human disease.

_The NOD mouse_

Diabetes in NOD mice develops rapidly; inflammation of beta cells due to infiltration of mononuclear cells occurs at approximately 4-5 weeks of age. Ensuing destruction of the beta cells and decreasing insulin concentrations lead to the presentation of frank diabetes within 12 to 30 weeks. The NOD mouse model has been used extensively in diabetes research because similarly to humans, the mouse MHC region largely influences the development of disease. As an indication to the importance of genetic heterogeneity, the NOD model consists of many genes related to susceptibility to autoimmunity. However the model differs from human Type 1 diabetes in that ketoacidosis is generally mild in NOD mice. While these mice can survive for extended periods of time without the administration of insulin, ketoacidosis is a serious condition in humans that can result in diabetic coma or death if left untreated. Additionally, the gender difference of animals developing diabetes does not reflect the findings of human studies. Studies involving NOD mice have focused on cell apoptosis in diabetic wounds and the immunological cascade consisting of T-helper type 2 cells, effector cells, and the contribution of cytokines.

_The BB rat_

Several strains of the BB rat exist, though only some are diabetes prone. For strains that develop diabetes, weight loss, hyperglycemia, and other diabetic complications occur at
approximately 12 weeks of age. Similarly to NOD mice, inflammation of pancreatic beta cells induces an autoimmune response and recruitment of T cells, B cells, macrophages, and natural killer cells. More in common with humans however, ketoacidosis in the BB rat is severe and fatal in the absence of administered insulin. The BB rat has been used in diabetic research to investigate the role of diet and a variety of viruses as possible environmental stimuli for the disease state\textsuperscript{60}.

**Animal models with specific known mutations or pathway defects**

In certain cases, specific known mutations or pathway defects influence the development of Type 1 diabetes and other autoimmune diseases. Animal models with such defects can be used to better understand pathways in human disease. Animals that exhibit specific defects in genes encoding important tolerance mediators for example can give insight to the role of these genes in humans\textsuperscript{63}.

**Humanized mouse models**

Humanized mouse models of type 1 diabetes are produced by introducing genes encoding MHC, T cell antigen receptors, and costimulatory molecules from humans. Specific aims of such models include modeling disease initiation and evaluating \textit{in vivo} immunomodulation by specific antigens presented in the context of humanized MHC and other genes\textsuperscript{63-65}. Unfortunately, humanized mouse models struggle to effectively reproduce human diabetes in the animal, particularly when human gene products interact with ECM and tissues that are not humanized\textsuperscript{63}. 
6.2: Animal models of Type 2 diabetes mellitus

Type 2 diabetes encompasses a heterogeneous group of disorders characterized by insulin resistance and impaired insulin secretion. This heterogeneity has led to a variety of animal models attempting to address the many facets of a complex disease. While some animal models emphasize insulin resistance, others predominately exhibit beta cell failure resulting in impaired insulin secretion. Different models will be more or less clinically relevant and extrapolation of results to the clinical setting must be undertaken cautiously.

6.2.1: Spontaneous animal models of Type 2 diabetes

The Kyoji Kondo (KK) mouse

Originally bred for large body size, this animal strain develops obesity and insulin resistance that is followed by mild hyperglycemia. The severity of the diabetic phenotype is highly dependent on food intake, and the model is useful in researching human obesity. Currently however, multiple strains of KK mice exist due to specific inbreeding or spontaneous mutations that are genetically and phenotypically different.

The Goto Kakizaki (GK) rat

The GK rat exhibits both insulin resistance and impaired insulin secretion having developed relatively stable hyperglycemia upon reaching adulthood. This model is useful for investigating some complications of diabetes as the rats develop renal lesions, structural changes in peripheral nerves, and retinal abnormalities similarly to humans.
6.2.2: Single gene mutation animal models of Type 2 diabetes

The Zucker (fa/fa) rat

Obesity is generally a fair predictor of Type 2 diabetes in humans. Thus, animal models of obesity have been developed in hopes of gaining insight into the disease. The Zucker rat is an obese strain that maintains normal blood glucose levels by counteracting insulin resistance with increased insulin secretion from pancreatic beta islet cells60.

The db/db mouse

In contrast to the Zucker rat, the db/db mouse strain fails to maintain the levels of insulin secretion necessary for normal blood glucose levels and thus quickly develops hyperglycemia60. The db/db mouse exhibits excessive eating, obesity, and elevated blood glucoses of 300-500 due to a defect in the leptin receptor. Similarly to the human condition, the mouse has a reduced ability to heal standard skin wounds in addition to decreased epidermal nerves67.

6.3: Animal models resulting from gene targeting and transgenic techniques

Recently, molecular biological techniques such as gene targeting have been used to develop new animal models. Knockout animals are produced by disrupting single genes within an embryonic stem cell. These defects are then transmitted along the germ cell line. Additionally, transgenic animals are formed when modified genes are incorporated into a host genome. Researchers have produced models by manipulating
various genes, including those that encode for insulin receptors or glucokinase. Resulting phenotypes include animals that exhibit varying degrees of insulin resistance and sensitivity and animals with varying blood glucose levels. Complications of these techniques arise when gene manipulation produces unexpected results. Many genes have different functions at various points in an animal’s life and may also influence physiological processes not involved in the disease process.

6.4: Selection of a diabetic animal model

With the variety of animal models representing both Type 1 and Type 2 diabetes, choosing an appropriate model for an experimental study requires careful consideration. To investigate internal wound healing adjacent to medical devices, it should first be determined which subset of diabetes is the investigative focus. While both Type 1 and Type 2 diabetics will need identical implants, such as glucose sensors and orthopedic implants, the mechanisms leading to the disease state and hyperglycemia differ. Type 1 diabetes is most easily produced and reproduced by the administration of toxins. The disease state requires less time to achieve and dosage can be customized to each individual animal. Animals generally express diabetes within several days of the last dose of drug. Type 2 diabetes is affected by a variety of factors, such as insulin resistance, impaired insulin secretion, obesity, and genetic and environmental factors. Because of this variety, investigations often begin with Type 1 animal models. Optimally, studies should be performed in a variety of diabetic animal models –
representing both Type 1 and Type 2 diabetes – for the purpose of comparison and confirmation of data.
Chapter 7: Medtronic Real Time Continuous Glucose Monitoring

Functional glucose sensors used for this doctoral research were provided by Medtronic. Three different percutaneous sensors were used: Medtronic MiniMed SOF SENSOR™, ENLITE SENSOR™, and ENLITE 2 SENSOR™. The sensor experiments performed involved mostly Sof and Enlite sensors (Figures 2-3), with a smaller subset of Enlite2 sensors (n=20). All sensors utilize the enzyme glucose oxidase, which is immobilized on the working electrode. Glucose oxidase catalyzes the oxidation of interstitial glucose to gluconic acid and the formation of hydrogen peroxide [Equations 1-3]. Voltage applied to the working electrode decomposes the hydrogen peroxide into electrons, oxygen, and hydrogen. This generated current is registered by the imbedded circuit board located in MiniLink™ transmitters where it is correlated to the concentration of glucose in the interstitial fluid. The reference electrode maintains a constant electrical potential between itself and the working electrode while the counter electrode dissipates electrons back to the surrounding environment.

Equation 1: \[ \text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconic acid} \]

Equation 2: \[ H_2O_2 \xrightarrow{\text{voltage}} 2e^- + 2H^+ + O_2 \]

Equation 3: \[ 2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \]

All sensors were used in in vivo experiments. The sensors were implanted percutaneously in the dorsum. Each sensor was then attached to a MiniLink™
transmitter. Two different transmitters were used for these experiments. GST transmitters (transmitting transmitters) power the glucose sensor, collect and store glucose data, and transmit the data using radio frequency to a computer. Current values are displayed continuously in real time as long as the transmitters are in close proximity to the computer and attached Comlink™ (Figure 4-5). iPro transmitters (recording transmitters) power the glucose sensor and continuously collect and record data. The transmitter is then removed periodically, and data are downloaded onto a computer using Medtronic software and a transmitter dock (Figures 4-5). Because data are not displayed in real time, iPro transmitters can record data without needing to be in close proximity to a computer. Both types of transmitters process continuous electrical signals received from the sensor as it reacts with interstitial fluid. An average glucose signal is computed every 5 minutes that correlates with the real-time interstitial glucose levels present in the local environment. Medtronic MiniMed has supplied this research with R&D MiniLink™ transmitters and software, transmitter charging stations, and a Comlink™. Conversion of electrical signals to glucose concentrations is possible via a proprietary algorithm. These data values are most accurate when sensors are properly
calibrated (at least twice daily, no further than 12 hours apart).

Figure 2: Medtronic SOF Sensor and attached MiniMed Transmitter; ©Medtronic

Figure 3: Medtronic Enlite Sensor; ©Medtronic

Figure 4: Medtronic MiniMed GST Transmitter (left) and iPro Transmitter (right) (~5 grams, 3.5cm x 3 cm x 1 cm) and iPro dock used for charging and downloading data from iPro transmitters; ©Medtronic
Figure 5: Transmitter Utility program for displaying real-time glucose data from GST transmitters. Comlink shown in upper right corner. ©Medtronic
Part II: ESTABLISHMENT OF A DIABETIC ANIMAL MODEL

Chapter 8: Development of a novel outcome-based dosing regimen for inducing Type 1 diabetes in a rodent model


8.1: Introduction

The procedure for inducing Type 1 diabetes in normoglycemic Sprague-Dawley (CD) rats is presented. A Type 1 diabetic model was chosen to avoid possible confounding variables as Type 2 diabetes encompasses a heterogeneous group of disorders influenced by genetic and environmental factors. A novel, outcome-based dosing regimen for administering the drug streptozotocin was developed. Streptozotocin (STZ) is a unique compound with broad-spectrum antibiotic, anti-neoplastic, and diabetogenic properties. Biochemically, STZ is an alkylating agent that modifies biological macromolecules and fragments DNA causing cell death. As an analog of N-acetylglucosamine (glucose), STZ causes selective pancreatic beta cell toxicity via uptake through GLUT-2 receptors leading to a state of hypo-insulinemia and diabetes. The drug can be administered IV or IP in a single large dose or multiple lower doses. Single large doses produce diabetes via direct cellular toxicity, whereas multiple lower doses...
produce diabetes via secondary autoimmune insulitis, mimicking human type-1 diabetes. Importantly, at low doses (40mg/kg) STZ is not broadly cytotoxic and is not known to cause immune dysfunction. Rats were given a variable number of streptozotocin doses to achieve a target blood glucose range of 350-600 mg/dL. Body weight and blood glucose levels were monitored throughout the experiment in order to validate the reproducibility and stability of the model.

8.2: Materials and methods

8.2.1: Materials

Streptozotocin was purchased from VWR International (Radnor, PA). A standard glucometer and glucose strips were used to monitor blood glucose levels (One Touch Ultra; LifeScan, Milpitas, Calif.).

8.2.2: Streptozotocin and sodium citrate buffer

Sodium citrate buffer (10 mM) was prepared by dissolving 147 mg of tri-sodium citrate in 49.5 ml of normal saline and adjusting the pH to 4.5 with approximately 0.5 ml of 1M hydrochloric acid. The citrate buffer was used fresh or stored at 4°C. Streptozotocin (VWR) aliquots were prepared by pre-weighing 32 mg streptozotocin (STZ) into 6ml plastic Vacutainers (Cardinal Health, Dublin, Ohio), wrapping the Vacutainers in aluminum foil to protect against light sensitivity, and storing at -20°C. For each experiment, pre-weighed STZ (32 mg) was mixed with 4 ml sodium citrate buffer (via direct injection of buffer into Vacutainer) to produce a final concentration of 8 mg/ml.
The STZ was then mixed by vigorously shaking the plastic vial for approximately 5-10 s until all powder dissolved into solution. Animal injections were then performed immediately after mixing to prevent degradation of the drug as it is relatively unstable in an aqueous environment. Residual STZ solution was discarded according to the safety protocols of our research institution.

8.2.3: In Vivo studies

This protocol was approved by the Duke University IACUC #A270-10-10. Male Sprague-Dawley (CD) rats (150-200g) were obtained from Charles River Laboratories (Raleigh, NC). A total of 24 rats were divided into two experimental groups, with 16 animals receiving streptozotocin injections and 8 animals receiving vehicle injections. The rats were fasted 8 hours prior to their first STZ injection (day 0) in order to facilitate uptake of the drug streptozotocin. Diabetes was induced in the experimental group of animals via daily intraperitoneal (IP) injections of STZ at 40 mg/kg for 3 consecutive days. Following STZ injections, rats were given drinking water supplemented with sucrose or glucose (15 g/l) for 48h to limit early mortality. STZ-induced pancreatic islet cell damage causes stores of insulin to be released, which can lead to severe hypoglycemia and diabetic coma. Approximately 48 hours after the third injection, 3-hour fasting blood glucose measurements were taken from the tail vein of the rats using a standard One Touch Ultra glucometer (LifeScan, Milpitas, CA). Fasting measurements were taken to control for inter-animal feeding variability. For the purpose of the
experiment, animals were considered diabetic when their fasting blood glucose exceeded 300 mg/dl (16 mM). However, all animals with a blood glucose level less than 350 mg/dl received a fourth dose of STZ. This was done to ensure that animals that were borderline diabetic (blood glucose 300-350 mg/dl) did not subsequently drop below our diabetic threshold over the course of the experiment. Approximately 48 hours later we repeated the fasting blood glucose measurements and injected animals with a fifth dose of STZ if their blood glucose was less than 350 mg/dl. This injection sequence was repeated until all animals in the experimental group reached target blood glucose range of 350-600 mg/dl. The animals in the non-diabetic group received three vehicle injections (citrate buffer) in the same method as described above. All animals were given food and water ad libitum and kept on a 12-hour light/dark cycle. Measurements of body weight and water intake were performed every other day to estimate nutritional status of the animals. Additionally, blood glucose was measured every two days for diabetic animals and every four days for non-diabetic animals.

**8.2.4: Statistical analysis**

Blood glucose and body weight data were normally distributed and therefore at day 0 and every 4th day subsequently the groups were compared using a student’s T test in JMP.
8.3: Results

8.3.1: Blood glucose

Fasting blood glucose measurements for the STZ (diabetic) and Vehicle (non-diabetic) groups were averaged for each day measured (Figure 6). A significant difference in the mean blood glucose for the diabetic group was observed when compared to the non-diabetic group for all days except day 0 (p < 0.001*). All animals in the diabetic group maintained a blood glucose level between 350-600 mg/dl for the entirety of the experiment. Average blood glucose for the diabetic group 14 days after achieving their initial hyperglycemic measurement was 466 ± 16 mg/dl (mean ± standard error, coefficient of variation = 0.15). The non-diabetic group maintained physiologic blood glucose, with an average of 105 ± 11 mg/dl (mean ± standard error, coefficient of variation = 0.10) 12 days after receiving the initial vehicle injection (Figure 6).
Figure 6: Average blood glucose (mg/dl) for both STZ (n=16, q2days) and Vehicle (n=8, q4days) rats measured by tail vein stick using a standard One Touch Ultra glucometer. Data are represented as mean ± standard error. There was a significant increase in the mean blood glucose for the STZ group when compared to the Vehicle group for all days except day 0 when compared by a Student’s t-test (p < 0.0001*).

8.3.2: Body weight

Body weight measurements for each animal were averaged by day for each group (Figure 7). We observed a significant difference in the mean body weight for the non-diabetic group when compared to the diabetic group for all days except day 0 (p < 0.001*). We also observed a difference in the trend in weight gain between the two groups, with the non-diabetic animals gaining considerably more weight than the
diabetics for the entirety of the survival period (Figure 7). It is important to note the diabetic animals did on average gain weight from day 4, 185 ± 4 g, to day 24, 205 ± 9 g (mean ± standard error). Average body weight on day 24 for the non-diabetic group was 349 ± 8 g (mean ± standard error).

Figure 7: Average body weight (g) for both the diabetic (n=16, q2days) and non-diabetic (n=8, q4days) rats. Data are represented as mean ± standard error. There was a significant difference in the mean body weight between the diabetic and non-diabetic groups for all days except day 0 when compared by a Student’s t-test (p < 0.0001*).
8.4: Discussion and conclusion

8.4.1 Streptozotocin and diabetes

Using this novel outcome-based dosing regimen, diabetes was induced in 100% of STZ-dosed CD rats, exceeding even the best results in the literature to date. Publications report as few as 50% of animals achieving target hyperglycemia after receiving a single large dose (100 mg/kg), with a maximum of 90% achieving target blood glucose using a fixed multiple low-dose regimen\(^6\). Additionally, outcome-based dosing minimized blood glucose variability as evidenced by the dose response curve (Figure 9). Dosing with STZ resulted in quick changes in blood glucose levels in the diabetic group, with 13 of the 16 animals requiring only 3 injections to reach target hyperglycemia. Two of the animals required 5 doses to reach the target range, and only 1 animal needed a 6\(^{th}\) dose. The rats tolerated the IP injections and none of the animals exhibited any signs of drug toxicity after receiving STZ. Over the course of the experiment no animals required exogenous insulin to prevent diabetic keto-acidosis (blood glucose > 600 mg/dL).

8.4.1.1 Diabetic rodent models

With the wide variety of diabetic animal models currently available, choosing an appropriate model for this study required careful consideration. Some of these models are reviewed below, with a brief description of the pros and cons of each.
Animal models that mimic diabetes mellitus include surgical excision or chemical destruction of the pancreas, repetitive inbreeding to select for diabetic traits, gene deletion “knockouts”, and transgenic “humanized” models. Type-1 diabetes is characterized by auto-immune destruction of the pancreatic beta islet cells leading to severe hypo-insulinemia, hyperglycemia, and life threatening ketoacidosis if left untreated. The effects of type-1 diabetes can be studied most simply in animals by administering toxins that damage the pancreas. Common examples of these toxins are streptozotocin and alloxan. Generally, chemically induced type-1 diabetes is the most reproducible means of generating a stable diabetic animal model. A chemically-induced disease state requires less time to achieve target hyperglycemia and dosage can be customized to each animal. Type-1 diabetes can also be selected for spontaneously through inbreeding over generations based on the presence of hyperglycemia. Two of the most common spontaneous rodent models for type-1 diabetes are the non-obese diabetic (NOD) mouse and the bio-breeding (BB) rat. However, both spontaneous models are fairly heterogeneous and contain numerous genes related to autoimmune susceptibility.

Type-2 diabetes is a multifactorial disorder affected by insulin resistance and/or impaired insulin secretion, obesity, genetic, and environmental factors. As with type-1 models of diabetes, spontaneous type-2 diabetes can be selected for via iterative
inbreeding. One example is the Goto Kakizaki (GK) rat that exhibits both insulin resistance and impaired insulin secretion upon reaching adulthood. This model is useful for investigating long-term complications of diabetes as the rats develop the classic microvascular triad of nephropathy\textsuperscript{64}, neuropathy\textsuperscript{65}, and retinopathy\textsuperscript{66}. In addition, gene deletion “knockout” models for type-2 diabetes include the db/db mouse, which exhibits excessive eating, obesity, and elevated blood glucose secondary to an absence of leptin receptors. Similar to humans with diabetes the mouse has a reduced ability to heal standard skin wounds\textsuperscript{67}. However, due to the complexity of human type-2 diabetes, no single animal model is currently capable of representing the disease. Depending on the specific genotype and phenotype selected for the disease may differ greatly and confound experimental results. A desire to avoid the heterogeneity associated with type-2 diabetes led our group to begin our investigation with a type-1 diabetic model.

For our research STZ was chosen because of its efficacy, reproducibility, and ease of control\textsuperscript{60}. As mentioned previously, chemical induction of diabetes provides is highly reproducible and does not confound experimental results. Although our study focused on a disease state most similar to human type-1 diabetes, type-2 models should be studied in the future to see if similar results are reproducible. Type-2 diabetics constitute
the vast majority of the population in which the prevalence of diabetes is expected to increase.

8.4.2 Body weight

Trends in body weight were monitored to ensure none of the animals experienced wasting secondary to severe hyperglycemia. The lack of substantial weight gain in the diabetic group is somewhat expected as the diabetic group modeled an uncontrolled type-1 diabetic state. Discrepancies in body weight between groups are likely evidence that physiologic changes outside of hyperglycemia were occurring in the diabetic group over the course of the experiment.

8.4.3 Conclusion

A novel-dosing regimen based on target blood glucose outcomes was used to induce Type 1 diabetes in a rat model. Whereas many studies administer multiple doses of streptozotocin, these protocols are not outcome-based, which results in a percentage of animals not becoming diabetic and being discarded from the study. Our method differs in that a variable number of streptozotocin doses are administered as needed, with 100% of our animals achieving the diabetic state. Rats received variable (at least 3) doses of streptozotocin at 40 mg/kg until they achieved target blood glucose levels of 350-600 mg/dl. This method differs from other dosing regimens noted in the literature in that it is outcome based instead of based on a specific number of injections. Using this method, we have been able to reliably induce stable diabetes in 100% of our wild-type rats.
Chapter 9: Effect of nitric oxide release on bacterial infection in an orthopedic healthy animal model

9.1: Introduction

Indwelling medical devices such as orthopedic implants account for 45% of nosocomial infections in the United States\textsuperscript{69}. Two percent of joint prostheses and five percent of fracture fixation devices become infected, yielding 112,000 cases of orthopedic implant-related infection\textsuperscript{29} and 25,000 device failures per year\textsuperscript{70}. Currently 5.7 million people in the US have an internal fixation device or an artificial joint\textsuperscript{71}, representing an immense population for whom this poses substantial risk. Despite treatment measures such as the use of laminar flow, systemic antibiotic prophylaxis and ultraviolet radiation\textsuperscript{72}, infection can still occur. Associated morbidity is substantial: infection is the second leading cause of joint replacement failure\textsuperscript{73} with treatment ranging from wide excision to extensive surgical revision to amputation. These measures can be both painful and costly, and cause significant expense, disfigurement, and psychological trauma.

In addition, patients who suffer from sub-clinical infections are also a concern since these infections are rarely identified through standard follow-up care and likewise are not included in morbidity outcomes. Conventional culture techniques are inadequate at
detecting the presence of bacteria on explanted devices and rates of sub-clinical infection may be far higher than historically thought\textsuperscript{74-75}. This common low-level colonization may contribute extensively to what is currently considered “aseptic” loosening, aggressive foreign body response, or impaired wound healing.

Much research has focused on targeting and modifying the implant-tissue interface in an attempt to reduce infection rates. Initial approaches, which hope to prevent contamination from progressing to established infection, include reduction of bacterial adhesion via alteration of implant biomechanical properties and coating of implants with surfactants, proteins, or polysaccharides. While these approaches have been successful in \textit{in vitro} studies, they have yet to show anti-infective efficacy \textit{in vivo}. Effective \textit{in vivo} treatments have consisted of coating orthopedic devices with chlorhexidine-silver sulfadiazine or minocycline-rifampin. Additionally, antibiotic-impregnated bone cement is currently in use for many joint replacement surgeries. However, as is the case with all antibiotic therapies, there is concern that the steady, slow release of antibiotics will lead to the development of resistant strains\textsuperscript{73}. Therefore, it is desirable to produce implants with anti-infective characteristics that do not rely on exogenous antimicrobials, but that instead enhance endogenous anti-infective capabilities of the immune system.
Nitric oxide (NO) is an endogenously occurring diatomic free radical with regulatory functions in the respiratory, gastrointestinal, cardiovascular, and other bodily systems\textsuperscript{76-79}. Additionally, NO produced by vascular endothelial cells induces the dilation of blood vessels and inhibits platelet activation\textsuperscript{80}. NO has also been firmly established as an effective antimicrobial agent\textsuperscript{81} which operates via modulation of cytokine production, cell proliferation, tissue remodeling\textsuperscript{82}, and angiogenesis\textsuperscript{83}. Because of the short half-life of NO in the body (<1 s), the effects of NO are almost exclusively localized to the area of NO synthesis and release.

While molecular NO itself does have bactericidal properties\textsuperscript{84}, it can also enhance the cellular immune response to infection. \textit{In vivo}, NO produced by macrophages can react with superoxide (O$_2^-$) to produce peroxynitrite (ONOO\textsuperscript{-})\textsuperscript{85}. ONOO\textsuperscript{-} then reacts with other molecules to produce hydroxyl and carbonate radicals that are highly reactive oxidative agents. These agents are critical in the destruction of contaminating microbes\textsuperscript{86}. Unfortunately, because NO synthesis is not initiated until long after exposure to immunostimulants, NO is the limiting reagent in this reaction\textsuperscript{87}. Thus, bacteria has time to create biofilms, which are stable colonies protected by a secreted polymer matrix. Biofilms are far more resistant to the immune response than contaminating bacteria and can withstand the eventual ONOO\textsuperscript{-} release produced from endogenous NO\textsuperscript{88}. Since O$_2^-$ is readily available, production of ONOO could occur.
before biofilm formation if NO was present earlier. Therefore, supplying exogenous NO immediately after contamination could enhance the local immune response and prevent the formation of biofilms and established infection\textsuperscript{89}.

Exogenous NO can be delivered in a highly adaptable manner by coating implants with a liquid sol-gel, which hardens to form a xerogel\textsuperscript{18,90}. These xerogels contain covalently bound diazeniumdiolates which spontaneously produce NO when exposed to hydrogen donors such as water\textsuperscript{91}. The NO release kinetics of these xerogels can be varied by changing either the type or the amount of NO donor precursors used. These include the aminosilanes (aminoethylaminomethyl)-phenethyltrimethoxysilane (AEMP3), \(N\)-(6-aminohexyl)aminopropyltrimethoxysilane (AHAP3), \(N\)-(2-aminoethyl)-3-aminopropylmethylidimethoxysilane (AEAP2), and \(N\)-[3-(trimethoxysilyl)-propyl]diethylenetriamine (DET3). The short- and long-term release of NO is greatest with a DET donor and least with an AEMP3 donor while AHAP3 and AEAP2 NO release levels range between the other two and are generally comparable\textsuperscript{18}.

These NO-releasing coatings have been shown to effectively resist bacterial adhesion of nosocomial pathogens to various implantable materials \textit{in vitro}\textsuperscript{91}. Presence of these xerogels noticeably improved resistance of stainless steel orthopedic devices to bacterial adhesion \textit{in vitro}\textsuperscript{92}. Since adhesion and biofilm formation are required for the
establishment of infection\textsuperscript{93}, prevention thereof may prove instrumental in reducing implant-associated infection rates. One study has shown significant decreases in infection levels in the presence of diazeniumdiolate-charged xerogel coatings on subcutaneous silicone implants as compared to uncoated controls\textsuperscript{94}. In addition, NO-releasing xerogels have been shown to have a number of other benefits, such as increasing vascularity within scar capsule tissue\textsuperscript{94}, possibly via augmented VEGF production\textsuperscript{95}, and improved tissue integration\textsuperscript{96}. These results suggest the possibility of improved monocyte transport, tissue oxygenation, and wound healing\textsuperscript{94}.

This study was designed to achieve three goals: first to establish a rabbit model of contaminated internal femur fixation in which localized infection can be reliably reproduced; second, to evaluate the anti-infective efficacy of nitric oxide (NO)-releasing xerogel coatings on orthopedic internal fixation devices; and third, to identify parameters at the time of implantation which have a measurable effect on the level of infection. The femurs of 12 adult rabbits were implanted with NO-releasing and control stainless steel compression plates and inoculated with \textit{Staphylococcus aureus} before closure. During implantation, variables such as hematocrit, temperature, and perfusion were measured. On the seventh post-operative day the implants were removed and the surrounding tissue was harvested, homogenized, serially diluted, and cultured to quantify infection at the implantation site. The study failed to reveal a significant
difference between levels of infection with the NO-releasing compression plates and the
control plates. However, these data did reveal a very strong correlation between the
temperature of the surrounding muscle at implantation and the bacterial load quantified
after seven days. This study underscores the important role of wound temperature
maintenance in the establishment of infection and may represent an easily manipulated
means by which to combat orthopedic implant-associated infection.

9.2: Materials and methods

9.2.1: Materials

Deionized water was obtained via reverse osmosis and maintained at a resistivity of
18M(ohm)/cm. Butyltrimethoxysilane (iso form; BTMOS) was purchased from Aldrich
(St. Louis MO). N-(6-aminohexyl)-aminoropyltrimethosysilane (AHAP3) was purchased
from Gelest (Morrisville, PA). Argon, nitrogen, and NO were purchased from National
Welders (Raleigh, NC). Barium chloride dihydrate was obtained from Sigma (St. Louis,
MO), lot 21K1219. Sulfuric acid, sodium chloride, potassium chloride, and monobasic
potassium phosphate were obtained from Malinckrodt (Paris, KY), lots 2876-9, 7581
KPME, 6858 KLNC, and 7100 KPSE respectively. Glycerol was obtained from Matheson
Coleman & Bell (Norwood, OH), lot 4H05. Dibasic sodium phosphate was obtained fro
Fischer Scientific (Fair Lawn, NJ), lot 720656. TSA plates and TSB were purchased from
VWR. Stainless steel compression plates were obtained as a gift from Synthes, Inc
(Paoli, IL). Meticillin-sensitive Staph aureus strain M1 used in this study was obtained
as a gift from Mark Shirtliff, Ph.D. of the University of Maryland. This strain was isolated at the University of Texas Medical Branch in Galveston from a human patient with active osteomyelitis and has been used in Dr. Shirtliff’s rabbit osteomyelitis model for other studies.

9.2.2: NO-releasing xerogel-coated implants

Twelve hole, 8mm wide stainless steel compression plates and 6-8mm x 2mm stainless steel self-tapping screws were donated by Synthes, Inc (Paoli, IL). Compression plates were trimmed using a hacksaw to a final length of ~1.5 cm. Each plate was then placed with a corresponding screw into a glass vial and autoclaved for sterility. Sol solutions were prepared by mixing 1.2 mL EtOH, 640 µl H₂O, and 110 µl 0.5M HCl, and then adding 1.28 ml BTMOS dropwise. After stirring overnight, 860 µl of AHAP3 was added slowly, and the final solution was further mixed for an additional 8 hours. The compression plates and screws were then coated with the sol solution, allowed to dry, and then coated once more. During drying, the pieces were spun to prevent sedimentation. The implants were stored at 55°C for 24 hours and then moved to a desiccator.

NO-releasing plates were created by addition of diazeniumdiolate NO donors in a 500 ml reaction chamber. The reaction chamber was first purged of atmospheric air with 5 atm Argon. The NO gas was incubated with solid KOH for at least 1 hour prior to
introduction into the gas chamber to reduce the presence of NO degradation products. The reaction chamber was then pressurized with NO at 5 atm for 2.5 days. At the end of this period the chamber was again flushed with 5 atm Argon. The reactive plates and screws were placed individually into glass vials, flushed with nitrogen, and stored at -80°C until used.

9.2.3: McFarland standards
1.175% barium chloride dihydrate (BaCl\(\cdot\)2H\(\cdot\)O) and 1% sulfuric acid (H\(\cdot\)SO\(\cdot\)) solutions were prepared from stock chemicals and deionized water by dilution. 0.5 McFarland standards were prepared by diluting 0.5 ml of the barium chloride solution with 99.5 ml of the sulfuric acid solution. The pH of the solution was then adjusted to 7.4 using NaOH. The standard was then aliquoted into glass screw-top vials, which were covered with Parafilm to maintain an air- and water-tight seal.

9.2.4: Bacterial husbandry
A single stab culture of S. aureus was obtained from the University of Maryland. The agar was warmed and 10 \(\mu\)l of the resulting liquid was inoculated into 2 ml of tryptic soy broth (TSB) and cultured at 37°C overnight. The colony was centrifuged at 2000 rpm for 10 minutes, and the supernatant was decanted. The pellet was rinsed with sterile PBS and then resuspended in 1 ml PBS and 3 ml 20% glycerol for a final 15% glycerol solution. This solution was stored at -80°C. Stationary phase cultures were grown from the -80 degree stock overnight at 37°C. A 100 \(\mu\)l aliquot of overnight cell
culture was then transferred into a fresh 2 ml TSB tube, which was incubated for ~6h at 37°C to obtain a log phase culture with a final concentration of ~1x10⁸ CFU/ml. This concentration was estimated via comparison with the 0.5 McFarland standard. Reproducibility of this standard was confirmed by serial dilution and cell plating of these cultures.

**9.2.5: In vivo studies**

The anti-infective efficacy of these NO-releasing implants was evaluated using male New Zealand-type white rabbits. Each adult rabbit was approximately 4kg at the time of implantation. The animal protocol was approved by the Duke University Institutional Animal Care and Use Committee (#A029-08-01) prior to initiation of these studies.

The rabbits were anesthetized with 120mg ketamine and 40 mg xylazine administered intramuscularly. Once sedated, both lateral thighs were shaved, and the rabbit was transferred to the operating table. Anesthesia was maintained throughout the procedure with 2% isoflurane in oxygen. The animal was positioned and the procedure was first performed on the right side. The operative field was prepared with three treatments of chlorhexidine followed by a final prep of isopropyl alcohol and sterile draping. Sterile technique was used throughout the procedure. A single 5-6 cm incision was made in the skin parallel to the femur as located via palpation of the greater trochanter proximally and the lateral epicondyle distally. The fascial plane between the
quadriiceps and hamstring muscles was located, and blunt dissection was used along this plane to expose the femur. At this point, a manual orthopedic drill was used to create a 2mm diameter hole in the cortex of the anterolateral surface of the femur. The wound was inoculated with 30 µl of a log-phase 1-2x10⁸ CFU/ml S. aureus suspension for a total inoculum of 3x10⁶ CFU. The inoculum was administered such that the femoral screw-hole was saturated with the suspension. The wound was clamped closed for 20 minutes after inoculation. Then, the wound was re-opened and either a control or an NO-releasing xerogel-coated plate was affixed to the femur using a corresponding control or NO-releasing xerogel-coated screw. The fascia overlying the muscle was then closed with a simple continuous stitch using 4-0 Vicryl suture. The skin incision was closed with interrupted 4-0 nylon sutures and then swabbed clean with isopropyl alcohol and hydrogen peroxide. This procedure was then repeated on the left side with the other type of plate (Figure 8).
Figure 8: Implantation procedure: A) Incision of skin directly above fatty streak demarcating the fascial plane between the quadriceps femoris and the hamstrings. B) Blunt dissection down this fascial plane reveals the femur. C) Coated plates are affixed to the femur with a single coated screw. D) Wounds are closed with interrupted 4-0 non-absorbable monofilament.

Before recovery, the animal was administered 10mg of flunixin subcutaneously. The same dose of flunixin was given once per day for the following two days. Animals were given access to food and water *ad libitum* and were house singly for the 7-day duration of their postoperative care. During the following week, animals were monitored daily for temperature, ambulation, stool amount and consistency, appetite, wound dehiscence or discharge, erythema, or other evidence of local or systemic infection.
After 7 days, the animals were once again anesthetized with 120mg ketamine and 40mg xylazine followed by 2% isoflurane in oxygen. The skin and fascial incisions were reopened, and the site of the plate exposed. A sterile cotton-tipped swab was then swiped once along the length of the wound on the surface of the femur from proximal to distal. This swab was placed into TSB for culture. The compression plate and screw were removed from the bone and also placed into TSB for culture. The deepest head of the vastus intermedius muscle was then identified anterior to the femur. This muscle belly was removed in its entirety via sharp dissection performed distally to proximally and divided into three portions. One portion was reserved for histological analysis. Another portion was weighed and dried to obtain an accurate wet-dry muscle mass ratio. The last portion was then weighed and reserved for quantitative microbiological techniques as described below. Finally, a length of femoral diaphysis including the implantation site was removed from the animal with a hacksaw and placed into TSB for culture. This procedure was performed on both sides, and the animal was sacrificed with sodium pentobarbital (Euthasol).

A subset of three animals was subjected to further testing during implantation and explanation procedures. First, a small amount of blood (~2ml) was drawn from the marginal ear vein after induction of anesthesia. This blood was separated into three heparinized centrifuge tubes and then spun for 10 minutes at 6000 rpm. The centrifuged
samples were then used to calculate the hematocrit of the animal as a measure of hydration status. The remainder of the blood was conserved and at the time of implantation was mixed with the *S. aureus* inoculum and allowed to clot before introduction into the implantation site. During implantation, two additional parameters related to local perfusion were measured for each site – the temperature and a laser Doppler flowmeter reading of the exposed biceps femoris muscle at the surface of the wound. The remainder of the implantation procedure was performed as described above. Similar parameters were measured at explantation, including the surface temperature and laser Doppler flow of the biceps femoris muscle as well as the laser Doppler flow reading for the intact vastus intermedius muscle prior to removal from the site. Otherwise, the explantation procedure was performed as stated previously.

**9.2.6: Quantitative microbiology**

Harvested muscle was homogenized in sterile PBS using autoclaved Pyrex mortar & pestle tissue homogenizers. The resultant solution was then subjected to serial dilution (6 iterations of 10x dilution), and 100 μl samples of each dilution were plated on TSA and incubated for 72 hours at 37 degrees C. After this time, the plate containing between 30 and 300 colonies was identified, and the CFU/ml calculated (Figure 9). This bacterial count was then used to determine the CFU/ml concentration of the original homogenate. The wet/dry ratio obtained from the second muscle sample was then used to calculate
the final endpoint of the study: the concentration of bacteria in CFU/mg dry mass that was present in the muscle at the time of explantation.

Figure 9: Quantification of bacterial load via serial dilution and plating. Note 10-fold dilution of bacterial from A to B and from B to C, etc. For this animal, the colonies on plate C are counted since $30 < n < 300$.

9.2.7: Histology

The reserved portion of the biopsied vastus intermedius muscle was immersion fixed in 10% buffered Formalin overnight and then transferred for storage in 70% EtOH. The samples were stained with PAS, blocked in paraffin, and then sectioned. Slides were viewed with light microscopy.
9.3: Results

9.3.1: Explantation microbiology

In the 7 postoperative days, all 12 animals recovered from surgery well without any signs of systemic infection. *S. aureus* was successfully recovered from all implantation sites, and only one subject showed evidence of bilateral contamination with other bacterial species. This subject was also the sole subject whose wounds dehisced and required an additional repair. Gross appearance of the implantation site wounds varied widely; some sites yielded large abscesses of purulent material and significant cutaneous erythema while others revealed no gross evidence of infection (Figure 10). Of note, the gross appearance of the wounds did not necessarily correlate with the calculated bacterial load present within the muscle biopsy.
Figure 10: Variable gross evidence of infection at time of explantation. Site A revealed no appearance of infection, but still yielded $>1 \times 10^3$ CFU/mg. Site B was extremely purulent upon reopening of the wound, but yielded an equivalent CFU/mg to that of site A (within one power of ten).

The bacterial load of the implantation sites varied widely for both the experimental and the control groups. The control subjects yielded a mean infection level of $2.9 \times 10^4$ CFU/mg with a standard deviation of $7 \times 10^4$ and a range of $2 \text{–} 2.5 \times 10^5$. The NO-releasing plates produced a mean infection level of $1.8 \times 10^4$ CFU/mg with a standard deviation of $2.4 \times 10^4$ and a range of $6 \text{–} 8.8 \times 10^4$ (Table 5). These results were not distributed normally (Figure 11). The control plates yielded a higher bacterial load than did the NO-releasing plates in six subjects; and the opposite was true in the other six (Figure 12). There was no correlation between the bacterial load obtained from one side of a subject and that on the contralateral side ($r = -0.0076, p = 0.9814$). No significant difference was found between the levels of infection for the two
groups when compared by an unpaired Wilcoxon ranked sum test (p = 0.5443; Figure 13).

Table 5: Bacterial load in CFU/mg

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.9e³</td>
<td>2.5e³</td>
<td>8.0e²</td>
<td>9.1e³</td>
<td>2.7e³</td>
<td>1.5e³</td>
<td>1.8e³</td>
<td>6.4e³</td>
<td>3.9e³</td>
<td>9.9e³</td>
<td>2.0e⁰</td>
<td>1.2e³</td>
</tr>
<tr>
<td>NO-releasing</td>
<td>3.3e³</td>
<td>1.7e³</td>
<td>2.0e³</td>
<td>5.0e³</td>
<td>2.2e³</td>
<td>3.1e³</td>
<td>1.9e³</td>
<td>1.3e³</td>
<td>9.9e³</td>
<td>9.3e³</td>
<td>6.0e⁰</td>
<td>2.2e³</td>
</tr>
</tbody>
</table>

Figure 11: Distributions of bacterial load. NO-releasing (left) and Control (right)
Figure 12: Bacterial load in CFU/mg for Control and NO-releasing plates, paired by subject. Control plates: $2.9 \times 10^4$ CFU/mg ± $7 \times 10^4$; NO-releasing plates: $1.8 \times 10^4$ CFU/mg ± $2.4 \times 10^4$ (Mean ± SD)
Figure 13: Bacterial load in CFU/mg for control vs. NO-releasing plates with mean and quartiles displayed.

9.3.2: Histology

Five subjects produced microscope slides that showed intact muscle tissue amenable to further analysis for both sides. These samples were reviewed for muscular structural integrity, inflammation, and presence of bacterial extracellular matrix formation. Of these subjects, three were chosen that were representative of the spectrum of infection levels seen.
Figure 14 shows a series of representative images from a single muscle biopsy in order of increasing amount of muscular destruction. Figure 14A shows a region of normal muscle tissue in which the muscle fascicles are large and plump with very little space and cellularity between them. Figure 14B shows a region in which leukocyte infiltration is just beginning to widen the spaces between the shrinking fascicles. Increasing cellularity is seen in Figure 14C, with the appearance of strands of extracellular material that stain positively with PAS. Finally, Figure 14D shows a region of biopsy in which muscular tissue has been completely destroyed and only inflammatory cells and extracellular matrix are seen.
Figure 14: Representative samples of muscle histology in order of increasing level of muscular deterioration. A) Furthest from the implant site, the muscle fascicles are close to normal (60% of area, average inter-fascicular distance 3.9 μm). B) In the next zone, the initial signs of inflammation are seen (15% of area; 6.75 μm). C) Close to the implantation site, fascicles begin to disintegrate (5% of area; 13.2 μm). D) Closest to the implant, no muscle fascicles are present and only inflammatory tissue is present (20% of area; 100 μm).

To quantify the subjective histological observations, the following system was used. The muscle sample was first divided into four zones; one zone representing all of the area in which the muscle fascicles were completely obliterated, one zone representing the surface of the muscle most adjacent to the implant in which fascicles could still be seen, one zone representing the muscle most distant from the implant, and one zone in between the latter two. The percentage of the slide represented by each zone was then
estimated. For example, if most of the biopsy contained intact muscle fascicles with little infective infiltration, then it would be estimated that ~80% of the slide was represented by the most distant zone. Each of the zones was then analyzed more closely to determine the average distance between muscle fascicles as a measure of inflammation and muscle destruction. The average distance was multiplied by the percent coverage of each zone, and then the scores for each zone were summed to produce a weighted index of inflammation. An intrafascicular distance of 100 μm was used for the areas in which fascicles were completely absent for a hypothetical maximum index of 100. As an example, for the site shown in Figure 14, the inflammation index is calculated as (0.6 x 3.9) + (0.15 x 6.75) + (0.05 x 13.2) + (0.2 x 100) = 24.02.

The inflammation indices ranged from 7.3 to 93.4 with a mean of 33.13, a median of 21.65, and a standard deviation of 32.45 (Table 6). The index compared favorably to the subjective determination of the level of muscle destruction. However, neither the gross appearance of the samples nor the calculated indices correlated well with the bacterial load measured in CFU/mg (r = 0.0440, p = 0.9341).
Table 6: Inflammation indices for the muscle biopsy histology slides reviewed.

<table>
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<tr>
<th>Subject</th>
<th>1A</th>
<th>1B</th>
<th>3A</th>
<th>3B</th>
<th>5A</th>
<th>5B</th>
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<tr>
<td>Inflammation Index</td>
<td>45.1</td>
<td>93.4</td>
<td>7.3</td>
<td>9.7</td>
<td>24.0</td>
<td>19.3</td>
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<tr>
<td>CFU/mg</td>
<td>9,900</td>
<td>3,300</td>
<td>20</td>
<td>800</td>
<td>22,000</td>
<td>2,700</td>
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</tbody>
</table>

9.3.3: Physiologic parameters

In three animals, additional physiologic parameters were measured to assess their correlation with the measured bacterial load at explantation. The variables measured were: a) the temperature of the surface of the surrounding quadriceps muscle at the time of implantation, b) perfusion as measured by Doppler flow of the surrounding muscle at the time of implantation, c) hydration status as measured by the hematocrit at the time of implantation, d) the temperature of the surface of the surrounding muscle at the time of explantation, and e) perfusion of the surrounding quadriceps and vastus intermedius muscles at the time of explantation (Table 7).
Table 7: Parameters measured in a subset of subjects to determine factors that may correlate with bacterial load. Hct – hematocrit; Temp I – temperature at the time of implantation; Doppler I – laser Doppler flow at the time of implantation; Temp E – temperature at the time of explantation; Q Dop E – laser Doppler flow of the quadriceps muscle at the time of explantation; VI Dop E – laser Doppler flow of the vastus intermedius muscle at the time of explantation.

<table>
<thead>
<tr>
<th>Subject</th>
<th>CFU/mg</th>
<th>Hct</th>
<th>Temp I</th>
<th>Doppler I</th>
<th>Temp E</th>
<th>Q Dop E</th>
<th>VI Dop E</th>
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<tr>
<td>10A</td>
<td>12,000</td>
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<td>63</td>
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<td>75</td>
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<tr>
<td>10B</td>
<td>88,000</td>
<td>0.38</td>
<td>34.6</td>
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<td>11A</td>
<td>2</td>
<td>0.36</td>
<td>36.2</td>
<td>49</td>
<td>35.3</td>
<td>86</td>
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</tr>
<tr>
<td>11B</td>
<td>6</td>
<td>0.36</td>
<td>36.4</td>
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<td>36.4</td>
<td>91</td>
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<tr>
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<td>28</td>
</tr>
<tr>
<td>12B</td>
<td>22,000</td>
<td>0.38</td>
<td>35.2</td>
<td>43</td>
<td>35.1</td>
<td>68</td>
<td>16</td>
</tr>
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</table>

The hematocrit was consistent among subjects. Muscle temperature at the time of implantation varied from 34.6 to 36.4 with a mean of 35.8. Laser Doppler flow readings at implantation varied from 43 to 63 with a mean of 53. Neither hematocrit nor laser Doppler readings correlated well with the ultimate bacterial load for the implantation sites ($r = 0.4589$ and $-0.1318$ respectively). Interestingly however, the muscle temperature at implantation showed a strong inverse correlation ($r = -0.9136$, $p = 0.0109$) with the eventual bacterial load (Figure 15).
Figure 15: Correlation between muscle temperature at implantation and the bacterial load calculated for that site.

Variables measured at the time of explantation cannot be manipulated to affect variance in the bacterial load, however they may be useful to observe the effects of infection level on the tissue. Temperature at the time of explantation was slightly lower than that at implantation with a range of 35.1 to 36.4 and a mean of 35.5. The perfusion of the quadriceps muscle was considerably better than that of the vastus intermedius muscle biopsy site, with a mean of 69.7 versus a mean of 25.0 (Table 7).

The temperature of the surrounding muscles at explantation did not correlate with the level of infection ($r = -0.0563$). The perfusion of the vastus intermedius muscle
did show an inverse correlation with bacterial load, which was almost significant ($r = -0.7981$, $p = 0.0571$). A significant inverse correlation was also shown for the perfusion of the surrounding quadriceps muscle and the infection level ($r = -0.9816$, $p = 0.0381$) (Figure 16). Interestingly, the perfusion of the vastus intermedius muscle also showed a very strong correlation with the temperature of the muscles at implantation ($r = 0.9610$, $p = 0.0023$) (Figure 17). This may suggest that the temperature of muscles at implantation has an effect on the development of infection, and the infection load subsequently affects perfusion to the surrounding musculature.
Figure 16: Correlation between Doppler perfusion readings for the external quadriceps muscle and the vastus intermedius muscle at the time of explantation and the calculated bacterial load.
Figure 17: Correlation between the muscle temperature at the time of implantation and the vastus intermedius Doppler perfusion readings at the time of explantation.

9.4: Discussion and conclusion

The NO-releasing xerogel chemistry used in this study has, in prior studies, been proven to reduce *in vitro* bacterial adhesion to various implantable materials\(^91,92,95\) and to reduce both *in vivo* foreign body response\(^93\) and infection rates\(^94\) involving subcutaneous silicone implants. Therefore, this study examined the efficacy of these coatings when applied to orthopedic devices *in vivo*. 
9.4.1: Anti-infective efficacy

No significant difference in ability to reduce bacterial load was observed between NO-releasing compression plates and control plates in this rabbit model using this particular NO coating formulation. This was surprising considering the data obtained from prior studies confirming anti-infective efficacy of these coatings. One possible explanation is the large amount of variability in the bacterial load between the implantation sites (2–3x10^5 CFU/mg). In the study evaluating the anti-infective properties of these coatings on subcutaneous silicone implants, the endpoint data ranged from 5–7x10^3 CFU/mg for the control group and from 0–6x10^3 CFU/mg for the NO-releasing group, a reduction in range by a factor of 100.

9.4.2: Sampling error

The lack of significant difference may also be influenced by the method in which samples were treated and obtained. In the previous subcutaneous implant model, the liquid inoculum of bacteria was pipetted into a subcutaneous skin pocket that was created for the implant. Any ensuing infection was thus well contained, and the model was highly reproducible in a localized site, resulting in ease of sampling. In the present model, the implantation site was within a large space between the femur and the surrounding muscle compartments. The bacterial inoculum was not well-localized to the compression plate and screw, but was able to flow freely in the space around the femur. Because of the difficulty in localizing the infection, the muscle biopsy taken may
not have been representative of the region of greatest bacterial burden. In fact, some of the subjects exhibited abscesses upon explantation that were either deeper or more distal than the muscle biopsy taken, or superficial to the fascia suture line. Attempts to better localize the bacterial inoculum were conducted in the final three subjects. The inoculum for these three animals was mixed with blood drawn from the subject intraoperatively. The blood/culture mixture was allowed to clot before introduction into the wound. This procedure prevented the free flowing of liquid inoculum, however the introduced clot was still difficult to localize reliably to the area of the implant. Thus, the variability in bacterial burden can possibly be reduced by exploring other methods of bacterial administration that can achieve reproducible localization.

9.4.3: Histology

The histology methods employed in this study were qualitatively interesting in the progression of muscle destruction that they revealed, however quantitatively, they failed to correlate well with the bacterial burden endpoint. This result could be due to sampling error in the biopsy taken for histology, the biopsy taken for microbiological analysis, or a combination of the two. PAS stain was used to specifically target extracellular matrix, or biofilm, secreted by the bacteria use; however the utility of this stain to the quantification of infection was unclear. Further clarification of the intent and utility of histological analysis should be established for future studies.
9.4.4: NO formulation

Using xerogel chemistry, it is possible to fine-tune the kinetics of nitric oxide release by varying the type and concentration of NO donor. The xerogels used in this study (20% AHAP3) are just one of many possible formulations of NO donor that can be employed with this technology. In particular, 20% AHAP3 was chosen because it was the formulation that produced the greatest reduction in *Pseudomonas* adhesion to glass\(^\text{18}\). However, it is possible that this formulation was simply too low-dose to have a significant effect on infection rates and levels in this particular model. Xerogels containing DET3 aminosilane, which releases greater quantities of NO in both the short- and long-term, may produce a more noticeable effect\(^\text{18}\).

9.4.5: Physiologic parameters

Once the large variability of endpoint data was identified, additional parameters were measured in subsequent subjects to identify variables that may correlate with implant site bacterial burden. These parameters included hematocrit, core temperature at the time of implantation, the temperature of the quadriceps muscle at the time of implantation, perfusion of the quadriceps as measured by laser Doppler flowmeter at the time of implantation, the temperature of the quadriceps muscle at the time of explantation, and perfusion of both the quadriceps muscle and the deep head of the vastus intermedius at the time of explantation. The temperature of the quadriceps muscle at the time of implantation was strongly inversely correlated with the eventual
bacterial burden at the implantation site. This suggests that one way to effectively reduce variability of bacterial load would be to more carefully control the temperature of the subjects and the implantation sites for the duration of the initial procedure.

The perfusion of the vastus intermedius and the rest of the surrounding quadriceps muscles at the time of explantation was also inversely correlated with the final bacterial load. This result suggests that a higher bacterial burden may reduce blood flow to the affected muscle tissue, or vice versa. While this perfusion parameter could not easily be manipulated to reduce endpoint data variability, perfusion readings could possibly represent an alternate or supplemental endpoint to measure in future evaluations of efficacy of these NO-releasing xerogel coatings.

The strong correlation between local temperature at the time of implantation and the resultant bacterial load represents a possible subject of future investigation. Little bench research has been performed that explores the relationship between temperature and surgical site infection in animal models. In the clinical realm, it is generally recognized that optimal tissue perfusion and oxygenation in the peri-operative period is facilitated by normothermia. Intraoperative hypothermia has generally been associated with increased risk of wound infection in both animal and human studies. In addition, the importance of thermal maintenance to infection prophylaxis has been emphasized in
clinical anesthesiology literature\textsuperscript{97, 98}. A reduction in core temperature by 1.9° C is associated with a threefold increase in infection risk in patients undergoing colon resection\textsuperscript{97}. Preoperative systemic warming reduces the incidence of surgical site infection in patients undergoing clean-wound breast, hernia, or varicose vein surgery according to a well-designed randomized controlled trial. This trial also showed that preoperative local warming at the surgical site was equally effective as systemic warming in infection prophylaxis\textsuperscript{99}. This study, supported by findings in the literature, clearly suggests the important role of local temperature in influencing infection of internal fixation devices.

\textbf{9.4.6: Differences in study design}

This study differed from prior studies in a number of ways, which could have possibly led to the lack of efficacy of the NO-releasing xerogels\textsuperscript{92, 94}. First, the animal model was changed from a rodent model (Sprague-Dawley rat) to a rabbit model. It is possible that the rabbit immune system differs from that of the rat and mouse, however it is difficult to determine what effects on bacterial burden this may have had. Second, the implant site was changed from the dorsal subcutaneous space to the surface of the femur. As mentioned previously, this modification may have led to difficulties in producing a localized infection and a consistent sampling technique. Third, the implant material was changed from silicone to stainless steel. Stainless steel has very different surface properties than does silicone, however NO-releasing xerogels have already been shown
in vitro to effectively reduce bacterial adhesion on orthopedic implants. Therefore the material of the implant may not be as much of a factor as the size and shape of implant. The surface area of the implant determines the level of NO coating and the eventual release kinetics and concentrations of NO. However, it is possible that even with a substantial surface area, the amount of NO release was still insufficient to deal with the bacterial burden. Finally, the strain of S. aureus differed between the two studies. The rodent model employed a standard, milder strain of S. aureus. This strain, when introduced into the rabbit model, was unable to reliably reproduce infection. Most subjects were able to clear the bacterial burden within the one-week time frame. The strain was then changed to a more virulent S. aureus that had been isolated from a human osteomyelitis patient. It is possible that the discrepant findings resulted from one or more of these or other factors. If the contributing parameters are identified and controlled, the anti-infective potential of the NO-releasing coatings may then be demonstrated.

9.4.7: Conclusion

The study described herein was an ambitious project designed to 1) establish a rabbit model of a contaminated orthopedic implantation site in which a localized S. aureus infection could be reliably produced, 2) use this model to evaluate the anti-infective efficacy of 20% AHAP3 NO-releasing xerogel coatings on orthopedic implants, and 3) determine controllable variables that may have an effect on the establishment of
implant-associated infections. This rabbit model was successful in reliably producing local infection of *S. aureus*. However, there may be aspects of the model and sampling techniques that contributed to excessive variability in the endpoint data. Possibly due to this variability, the study failed to show a significant difference in bacterial load between NO-releasing and control xerogel coatings. Additionally, the formulation used may not have had optimum nitric oxide release kinetics to adequately combat the establishment of infection. Further exploration of this NO-releasing xerogel technology with different formulations is certainly warranted, as prior studies do suggest that augmentation of endogenous NO can be an effective way to combat implant-associated infection. Finally, it was clearly demonstrated that the local temperature of the tissue surrounding the implant at the time of implantation surgery was strongly correlated with the ultimate level of infection established at that site. This finding suggests a possible source of variability in the endpoint data, and also reveals an association that may be clinically relevant. It is worth exploring this relationship further, as wound temperature is a factor that could be easily manipulated in a clinical setting to provide effective non-pharmaceutical implant-associated infection prophylaxis.
Chapter 10: Investigation of infection response in an orthopedic diabetic animal model


10.1: Introduction

In 2007, the American Diabetes Association estimated nearly 25 million people were affected by diabetes mellitus in the United States¹. With respect to the field of orthopedic surgery, the impact of diabetes is substantial. Diabetes associated diseases treated by orthopedists include: non-healing ulcer debridement, limb and digit amputations, Charcot (neurogenic) arthropathy, Dupuytren’s contracture, flexor tenosynovitis (trigger finger), and carpal tunnel syndrome. Diabetic patients are also at an increased risk of bone fractures²,³ due to changes in the quality and quantity of their bone⁴. The vast majority of these pathologic fractures have to be fixed surgically and involve internal hardware. Therefore, as the incidence of diabetes increases, the incidence of patients requiring indwelling hardware will also increase.

Currently, over 2.5 million orthopedic surgical procedures are done annually involving implantable materials⁵. Indwelling devices are associated with a variety of complications, with one of the most important being infection⁶. In the field of
orthopedic surgery, device-related infections are associated with extensive soft tissue
damage and osteomyelitis\textsuperscript{106}. Surgical implant infections are generally difficult to
manage, often requiring prolonged antibiotic therapy and revision operations\textsuperscript{34, 107, 108}. Of
the estimated 2 million fracture fixation devices implanted annually in the U.S,
approximately 5\% become infected\textsuperscript{34}, with each infection costing the healthcare system
about $15,000(Table 2). \textit{S. aureus} is the main offender causing greater than 40\% of these
hardware associated infections\textsuperscript{109}.

Orthopedic surgery patients with diabetes mellitus have increased rates of peri-
operative complications when compared to non-diabetic patients\textsuperscript{110, 111}. Several large-
scale case-control studies have looked at diabetic patients’ increased odds of developing
infections after orthopedic surgery\textsuperscript{46, 47, 112-114}. These studies report post-operative
infection rates in diabetics between 7-10\%, with a 1\% rate of infection in non-diabetics.
In patients undergoing joint arthroplasty, the odds of acquiring infection post-
operatively were 1.7 times higher in diabetic patients as compared to non-diabetics\textsuperscript{113}. In
cases of uncontrolled diabetes, the odds of acquiring infection increased to 2.3 times
higher for the diabetics\textsuperscript{47}. Although this relationship between diabetes and post-
operative infection has been reported in numerous retrospective clinical papers, there
are currently no models of post-operative infections in diabetic animals after hardware
implantation.
Development of a model to quantify the bacterial burden in type-1 diabetic rats and controls after undergoing hardware implantation and inoculation with \textit{S. aureus} is presented. A novel dosing regimen of streptozotocin based on a target blood glucose of 350-600 mg/dl was used to induce type-1 diabetes in this study. Stable hyperglycemia was maintained for at least 21 days. Two weeks after achieving the target hyperglycemia, control and diabetic rats received one stainless steel fracture plate and fixation screw on each anterior femur. One implant site per rat received an inoculum of $10^7$ CFU of methicillin-sensitive \textit{Staphylococcus aureus}. After seven days, quantitative bacterial count was performed at explantation, and no cross-over of bacteria was detected from the inoculated side to the non-inoculated side.

10.2: Materials and methods

10.2.1: Materials

Deionized water was obtained via reverse osmosis and maintained at a resistivity of $>18\Omega\text{cm}$. Isobutyltrimethoxysilane (BTMOS) was purchased from Aldrich (St. Louis, MO). N-(6-aminohexyl)-aminoropyltrimethosysilane (AHAP3) was purchased from Gelest (Morrisville, PA). Barium chloride dehydrate was obtained from Sigma (St. Louis, MO). Sulfuric acid was obtained from Malinckrodt (Paris, KY). Glycerol was obtained from Matheson Coleman & Bell (Norwood OH). Tryptic soy agar (TSA) plates and trypticase soy broth (TSB) were obtained from VWR International (Radnor, PA).
10.2.2: Stainless steel implants

Twelve hole, 8mm wide stainless steel compression plates were donated by Synthes, Inc (Paoli, IL). Implants were prepared by cutting these plates into segments approximately 1 cm long and containing two screw holes using a Dremmel Multipro Rotary Tool. Edges were filed smooth after sectioning. Self-taping stainless steel cortical screws (2.0 mm diameter, Synthes) were prepared by cutting to an approximate length of 4-5mm. All instruments and hardware were sterilized in an autoclave prior to surgery.

10.2.3: McFarland standards

1.175% barium chloride dehydrate (BaCl2*2H2O) and 1% sulfuric acid (H2SO4) solutions were prepared from stock chemicals and deionized water by dilution. McFarland Standards of 0.5, 1.0, and 5.0 were prepared by diluting 0.5 ml, 1.0 ml, and 5ml of the barium chloride solution into 99.5 ml, 99.0 ml, and 95.0 ml of the sulfuric acid solution, respectively. Using NaOH, the pH of the solutions was adjusted to 7.4 and the standards were then aliquoted into glass screw-top vials. Parafilm was used seal the vials.

10.2.4: Streptozotocin preparation

Sodium citrate buffer (10 mM) was prepared by dissolving 147 mg of tri-sodium citrate in 49.5 ml of normal saline and adjusting the pH to 4.5 with approximately 0.5 ml of 1M hydrochloric acid. The citrate buffer was used fresh or stored at 4°C. Streptozotocin (VWR) aliquots were prepared by pre-weighing 32 mg streptozotocin (STZ) into 6ml
plastic Vacutainers (Cardinal Health, Dublin, Ohio), wrapping the Vacutainers in aluminum foil to protect against light sensitivity, and storing at -20°C. For each experiment, pre-weighed STZ (32 mg) was mixed with 4 ml sodium citrate buffer (via direct injection of buffer into Vacutainer) to produce a final concentration of 8 mg/ml. The STZ was then mixed by vigorously shaking the plastic vial for approximately 5-10 s until all powder dissolved into solution. Animal injections were then preformed immediately after mixing to prevent degradation of the drug as it is relatively unstable in an aqueous environment. Residual STZ solution was discarded according to the safety protocols of our research institution.

10.2.5: Bacterial husbandry

A methicillin-sensitive strain of *S. aureus* derived from a previously resistant strain of Colindale (termed COL, thought to be strain 9204) was used in this study and was obtained as a gift from Vance Fowler, MD, MHS, Infectious Disease, Duke University. Frozen stocks were created by placing 100 μl inoculum of stationary phase culture into fresh 2 ml TSB tubes. After incubating overnight at 37°C to 0.5 McFarland Standard, the colony was gently centrifuged at 2000 rpm for 10 minutes and the supernatant decanted. The pellet was then resuspended in 1 ml phosphate buffered solution (PBS, Ca and Mg free Dulbecco’s, Sigma, Ayrshire, KS) and 3 ml 20% glycerol for a final glycerol concentration of 15%. Aliquots were placed into 1 ml cryotubes and stored at -80°C. Inoculums for *in vivo* studies were reconstituted from the stored -80°C stock. Stationary
phase cultures of MSS COL strain were grown from the -80°C stock overnight at 37°C in an 8 ml TSB tube. A 100 μl aliquot of overnight culture was then transferred into a fresh 8 ml TSB tube and incubated at 37°C for 5 hours to obtain a log phase culture. This equated to approximately 2 x 10⁸ CFU/ml as estimated by comparison to the 1.0 McFarland standard. Reproducibility of this standard was confirmed by serial dilution and plating of cultures.

10.2.6: In vivo studies
This protocol was approved by the Duke University IACUC #A270-10-10. For the experiment, male CD rats (150-200g) were obtained from Charles River Laboratories (Raleigh, NC). The rats were fasted 8 hours prior to their first STZ injection (day 0). Diabetes was induced in the experimental group of animals as previously described. The animals in the non-diabetic group received vehicle injections (citrate buffer). All animals were given food and water ad libitum and kept on a 12-hour light/dark cycle. Measurements of body weight and water intake were performed every other day to estimate nutritional status of the animals. Blood glucose measurements were taken from the tail vein of the rats using a standard One Touch Ultra glucometer (LifeScan, Milpitas, CA).

10.2.7: Femur implant and infection survival procedure
Fourteen days after developing diabetes, animals were brought into the preparatory room, and anesthesia was induced with 2% isoflurane in oxygen. Once a surgical plane
of anesthesia was established, the hindlimbs and lower back of the animals were shaved, and the animals were pre-scrubbed with alcohol pads. The animals were then transferred to the operating room and placed prone on the operating table. A rectal thermometer was inserted and the intra-operative temperature of the animals was monitored for the entirety of the operation. Any bouts of hypothermia were regulated with a heating pad under the operative field and a space heater under the operating table. The right hindlimb was prepared for surgery first with three treatments of chlorhexidine gluconate soaked sterile gauze. The animals were then draped with sterile towels and the chlorhexidine was allowed to dry before the incisions were made. For each animal we operated on the right hindlimb first and closed the wound completely before moving on to the left side. Using scissors, a 1-inch incision was made on the lateral aspect of the right thigh parallel to the femur (between the hip and the knee using the greater trochanter and patella as surface landmarks). A plane was then developed between the flexor and extensor musculature using a combination of sharp and blunt dissection. The antero-lateral aspect of the femur was exposed and the femur was stabilized in slight external rotation using a large grasping clamp. A pilot hole was drilled through the proximal cortex of the femur using a 1.5 mm diameter drill bit (Synthes). The fracture plate was then placed directly onto the femur and fixed into place with one cortical screw. The screw was inserted far enough to stabilize the plate, but care was taken not to over-tighten the screw to prevent femur fracture. After plate
fixation, the right femur and implant were inoculated by pipetting 10 µl of MSSA Col at a concentration of 2 x 10^8 CFU/ml (1.0 McFarland Standard) directly onto the fracture plate. The wound was then closed in two layers, first by suturing the extensor and flexor muscle fascia back together with 4-0 Maxon synthetic absorbable sutures in a simple continuous pattern. The skin incision was closed with either 3-0 or 4-0 polypropylene or nylon monofilament suture in a simple interrupted pattern. The S. aureus inoculation was allowed to incubate in situ between the fracture plate and overlying extensor musculature until the left femur implant was complete. After skin closure, the surgical draping was taken down, and the left hindlimb was prepared and draped with a new set of sterile towels in the same manner as described above. With a new set of sterile instruments, the entire implant procedure on the left side was then performed as described except that no bacterial inoculation occurred. After skin closure the animals were allowed to breathe 100% oxygen until spontaneous movement was detected. The animals were then returned to their cage and monitored until sternal recumbency was maintained before being returned to the Vivarium.

10.2.8: Survival period

All animals were maintained according to the standard of care guidelines established by the Duke University IACUC. All animals received a dose of 2.5 mg/kg body weight Flunixin meglumine administered subcutaneously immediately post-op and then daily
for three post-operative days. Daily monitoring for wound dehiscence, discharge, erythema or other evidence of local or systemic infections was conducted.

10.2.9: Non-survival surgery

Seven days after the implant procedure (approx. day 25), tissue was harvested at the site of bacterial inoculation (right hindlimb) and the non-inoculated control (left hindlimb). The rats were anesthetized as described above with 2% isoflurane in oxygen. When necessary, both legs were re-shaved before being cleaned with alcohol in the preparatory room. The animal was then placed in the prone position on the operating table. The order of explantation was opposite that of implant, with the left hindlimb being done first followed by the right side. This was done to prevent any contamination of the non-inoculated side with the inoculated side. The area around the left incision was prepped and draped as described above. The skin sutures were taken down and a new incision was made directly over the previous wound using heavy scissors. The femur was then exposed through blunt dissection and take down of the previous deep fascial closure. The vastus lateralis muscle of the animal was then carefully removed with dissection scissors. Only the muscle directly overlying the implant was taken by cutting the tissue in line with the ends of the plate. Therefore, the explanted musculature roughly approximated the size and shape of the two-hole fracture plate. The muscle was then divided into three segments with the dissection scissors. The proximal segment was used for a wet:dry weight analysis, the middle segment preserved for histology, and the
distal segment used for bacterial quantification via plate culture method. The screw and plate were then removed and placed in a sterile glass vial and saved for biofilm quantification. The left side was then packed with sterile gauze to prevent hemorrhage while the contralateral side was explanted. The skin was then closed in a simple running pattern with 3-0 or 4-0 polypropylene or nylon monofilament suture. The right side was then prepped and draped and the explantation procedure was conducted in exactly the same manner as described above. While under anesthesia, the animals were euthanized with a 3 ml intra-cardiac infusion of saturated (> 3M) KCl.

10.2.10: Quantitative microbiology
As described above, the harvested musculature was divided into three portions with dissection scissors. The tissue being used for wet:dry calculation was weighed wet and then dried at 50°C for 24 hours. The muscle was then weighed again and the wet:dry weight was calculated. The specimen taken for culture was weighed, minced with dissection scissors, and then homogenized in sterile PBS buffer (10X v:w). The resulting homogenate underwent six serial 10X dilutions and 100 µl of each dilution was plated on TSA plates and incubated for 72h at 37°C. After 72h, the plates containing between 30-300 colonies were counted for absolute number of CFU’s. The CFU/mg dry tissue of the original sample was then calculated.
10.2.11: Biofilm assay

Recovered stainless steel implants and cortical screws were transferred into a sterile Falcon 24-well plate and washed 6X with 1ml sterile PBS. Hardware was then transferred into a 3 ml glass red-topped Vacutainer containing 1ml TSB + 1% glucose and sonicated for 5 min at 37°C to remove adhered biofilm. Next, three 300 µl aliquots of the sonicated broth were transferred into separate wells on the Falcon plate and incubated overnight at 37°C. After incubation, the broth was aspirated and each well was washed 3X with 1 ml sterile PBS. Three hundred µl Crystal Violet (1% w:v in sterile PBS) was added and incubated for 5 min at room temperature. The stain was then aspirated and wells were washed three times with sterile PBS. Finally, 300 µl of glacial acetic acid was added to the wells to re-suspend the crystal violet stain and absorbance was immediately measured at 595nm using a Genios spectrophotometer running Magelan software (Tecan, Männedorf, Switzerland).

10.2.12: Study design

A total of 24 rats were divided into two experimental groups, with 16 animals receiving STZ injections and 8 animals receiving vehicle injections. All animals received bilateral femur implants. The right hindlimb of each rat was inoculated with MSSA strain COL while the left hindlimb served as the non-inoculated control. Therefore, there were a total of 4 different rat hindlimb groups. The non-diabetic (ND) non-inoculated (N) data served to quantify infectious burden without direct inoculation in non-diabetic rats. The
diabetic (DM)-N data were compared to the ND-N data to determine whether having diabetes alone in the absence of direct inoculation increases infectious susceptibility (as measured by bacterial concentration at site of operation). The ND-inoculated (I) data served as a control to see if bacterial inoculation produces a measurable infection in a non-diabetic animal. The DM-I data were compared to the ND-I group to see if having diabetes has an effect on the immune system’s ability to fight and clear infection. In addition, hardware was implanted bilaterally into control rats without inoculating the right hindlimb with \textit{S. aureus}. These data were compared to the ND-N data to see if measurable crossover of bacteria occurs between the inoculated and non-inoculated hindlimbs.

\textbf{10.2.14: Statistical analysis}

Infectious burden in the muscle biopsies was determined by quantifying the final CFU/mg dry tissue. The median, 25\textsuperscript{th}, and 75\textsuperscript{th} quartile CFU/mg tissue for the diabetic and non-diabetic groups were calculated using JMP software (SAS, Cary, NC). The data for both the inoculated and non-inoculated hindlimbs of both the diabetic and control groups did not follow a pattern normal distribution, and therefore the data were compared using non-parametric statistic in JMP. All descriptive statistical graphs (box and whisker plots) were created using SAS/STAT software. Additionally, the biofilm assay data was also not normally distributed and therefore was analyzed via non-parametric statistics. Blood glucose and body weight data were normally distributed.
and therefore at day 0 and every 4th day subsequently the groups were compared using a student’s T test in JMP.

10.3: Results

10.3.1: Quantitative microbiology

Infectious burden was measured by quantifying the number of colony forming units (CFU) of *S. aureus* 72h after muscle explantation (Figure 18). A significant difference in infectious burden as measured by bacterial quantification (CFU/mg dry tissue) in the inoculated limb (right) between the diabetic and non-diabetic groups was observed when compared by Wilcoxon rank-sum test. (*p* = 0.0003*, Figure 18). The median number of CFU/mg dry tissue in diabetic group was approximately 6 orders of magnitude greater than in the non-diabetic group (Table 8). Additionally, the diabetic group had a higher median CFU/mg dry tissue than the non-diabetic group in the non-inoculated limb (left), although this difference was not significant (Figure 18, Table 8).

As expected, when the bacterial burden for the inoculated vs. non-inoculated limb within each group (diabetic vs. non-diabetic) was compared, a significant increase in the CFU/mg dry tissue for the inoculated limb was detected (Table 8). To analyze if crossover of bacteria from the inoculated to non-inoculated limb was occurring, orthopedic plates were implanted bilaterally in control rats without inoculating either side. No significant difference in the CFU/mg dry tissue for the non-inoculated limb in
the diabetic group vs. the control group was observed when analyzed by Mann-Whitney U test (p = 0.64).

Table 8: Quantitative values for the infectious burden in the inoculated and non-inoculated limbs for both the diabetic and non-diabetic groups (data represent median CFU/mg dry tissue). A significant increase in the infectious burden in the diabetic group for the inoculated side (right) was detected when compared to the non-diabetic groups using a Wilcoxon rank-sum test (p = 0.0003*). No significant difference in the infectious burden between groups for the non-inoculated side was detected when compared using a Wilcoxon rank-sum test (p = 0.0682). A significant difference in infectious burden between the inoculated and non-inoculated side for both diabetic and non-diabetic groups was detected when compared using a Wilcoxon rank-sum test (p < 0.0001* and p = 0.0017*, respectively).

<table>
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<tr>
<th></th>
<th>Diabetic</th>
<th>Non-diabetic</th>
<th>p</th>
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<tbody>
<tr>
<td>Inoculated</td>
<td>$3.4 \times 10^{10}$</td>
<td>$7.2 \times 10^{4}$</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Non-inoculated</td>
<td>$2.6 \times 10^{5}$</td>
<td>$1.3 \times 10^{3}$</td>
<td>0.0682</td>
</tr>
<tr>
<td>p &lt; 0.0001*</td>
<td>P = 0.0017*</td>
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Figure 18: Infectious burden in the inoculated (left) and non-inoculated hind limb (right) for the diabetic ($n = 14$) and non-diabetic ($n = 7$) groups quantified by plate culture method. Box plot data represent median, 25th percentile, and 75th percentile, with whiskers representing minimum and maximum values. Vertical axis is logarithmic base 10 scale, with values representing power of the base. There was a significant difference in the concentration of bacteria cultured for the inoculated hind limb between the diabetic and non-diabetic groups ($p = 0.0003$). There was not a significant different detected in the infectious burden for the non-inoculated hind limb between the diabetic and non-diabetic groups ($p = 0.0682$).

Of the 24 rats in this experiment, one rat from the diabetic group died prematurely of femur fracture and one rat from the non-diabetic group died prematurely secondary to natural causes. Additionally, the culture plates of one rat in the diabetic group became
contaminated and were not included in the final data analysis. Therefore, the final data analyzed were n=14 and n=7 for the diabetic and non-diabetic groups, respectively.

10.3.2: Biofilm formation

The degree of biofilm formation on the implanted hardware was quantified using a technique originally described in 1985 by Christensen et al\textsuperscript{115} and modified by Antoci Jr. et al\textsuperscript{116}. A significant difference in the normalized absorbance (Figure 19) for the inoculated hardware between the diabetic and non-diabetic groups was observed when compared by Wilcoxon rank-sum test (p = 0.0017*, Table 9). Differences in variability in the diabetic and nondiabetic groups can most likely be attributed to the severity of the disease state in diabetic animals, in particular, variable levels of hyperglycemia, which influences bacterial adherence and biofilm formation.

Table 9: Normalized absorbance data of biofilm adherence to the inoculated hardware. A significant increase in absorbance for the diabetic group vs. the non-diabetic group was detected when compared by Wilcoxon rank-sum test (p = 0.0017*). Data represents median normalized absorbance (25\textsuperscript{th}, 75\textsuperscript{th} percentile).

<table>
<thead>
<tr>
<th>Normalized Absorbance</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
<th>p = 0.0017*</th>
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<tr>
<td></td>
<td>1.37 (1.11, 2.06)</td>
<td>0.92 (0.88, 0.94)</td>
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</table>
Figure 19: Absorbance of hardware as measured by indirect staining of biofilm adherent to the fracture plate and screws at the time of explantation surgery in both the diabetic and non-diabetic groups. Absolute absorbance of crystal violet was measured spectrophotometrically @570 nm. Box plot data represent median, 25th, 75th percentiles, with whiskers representing ± 1.5 times the interquartile range of the inoculated limb of each group after normalization to the non-inoculated limb. The dashed red line has a value of 1 and represents the non-inoculated limb data. There was a significant increase in the amount of biofilm detected in the inoculated limb of the diabetic rats when compared to non-diabetics as measured by a Mann-Whitney U test (p = 0.0017*).
10.4: Discussion and conclusion

10.4.1: Bacterial quantification

The model developed in this study was able to consistently reproduce the primary outcome of quantifying S. aureus infections at the surgical site 7 days after plate implantation and bacterial inoculation. The data showed a decreased ability to clear post-operative infections, which aligns nicely with the abundant literature on physiologic dysfunction in diabetic rats. The effects of diabetes are multifactorial and include immune dysfunction, microvascular damage, impaired mass transport, delayed wound and bone healing, and diabetic neuropathy. Specifically for this study, immune dysfunction and delayed wound healing provide mechanisms to explain the increased infectious burden seen in the diabetic rats.

10.4.1.1 Immune dysfunction in diabetics

Diabetes represents a mild immunocompromised state that predisposes individuals to post-operative infections. In diabetics, it has been shown that medically important immunologic defects occur almost exclusively in the cellular innate immune system, with multiple dysfunctions in both neutrophils and macrophages. Neutrophil dysfunction includes altered activation, increased cell adhesion, altered chemotaxis, and decreased phagocytosis; while macrophage dysfunction includes decreased cell number, immunogenicity, phagocytic rate, and antigen presenting ability. A summary of these and other immunologic dysfunctions found in diabetic animals can be
found in Table 3. These *in vitro* findings are consistent with the results of this study and further indicate that immune dysfunction in diabetic patients increases their post-operative infectious burden. Importantly, both studies mentioned that strict glucose regulation improved immune function in the diabetic animals, which suggests that the degree of immune dysfunction is proportional to blood glucose. However, one clinical study analyzing short term control of diabetes peri-operatively for patients undergoing orthopedic surgery found no significant reduction in surgical site infections when HbA1c < 7%\textsuperscript{118}. The authors concluded that blood glucose levels might not directly correlate with immunologic dysfunction. Therefore, the results of this study necessitate further experimentation as to whether glucose control via treatment with exogenous insulin has effects on infectious burden in our model. Importantly, the rodent immunologic dysfunction described here must be taken in proper context, as the decreased immune response to infection seen in diabetic humans could be different\textsuperscript{119,120}.

10.4.1.2 Abnormal healing around implants in diabetics

Over 100 known physiologic factors have been discovered that contribute to wound healing deficiencies in individuals with diabetes. These include but are not limited to decreased or impaired growth factor production, angiogenic response, collagen accumulation, quality of granulation tissue, and delayed bone healing\textsuperscript{121}. Delayed bone healing is important in the case of fracture plate fixation as mal-union or non-union can lead to hardware failure and subsequent revision surgery. Vascular pathology is also a
major player in delayed wound healing and leads to reduction in capillary size, arteriolar hyalinosis, and thickening of the basement membrane, with the major consequences being hypoxia and decreased angiogenesis. Decreased blood flow to the site of hardware implantation predisposes individuals to surgical site infections and provides an additional mechanism to explain why diabetics would have an increased post-operative infectious burden.

Although the surgical model presented in this study is novel, other groups have investigated in vivo infection susceptibility to S. aureus in diabetic rodents. It has been shown that when exposed to direct injection of S. aureus into their hindpaw, both type-1 and type-2 diabetics (NOD and leptin receptor knockouts, respectively) had a significantly higher mean bacterial burden when compared to non-diabetics. These data correlate nicely with the in vitro data described previously. Taken together, all of these studies support our hypothesis that diabetics have a decreased ability to fight and clear infections in the post-operative period.

10.4.2: Biofilm formation

Biofilm formation is especially relevant to this study as its presence makes treating and clearing orthopedic implant infections extremely difficult if not impossible. Due to S. aureus' notorious ability to generate biofilms on orthopedic hardware, the degree of biofilm formation on the fracture plate and screw was quantified indirectly. Results
showed a significant increase in the ability of *S. aureus* to form a biofilm on the implanted hardware in the diabetic animals. Because the majority of biofilm formation occurs during the early post-operative period, the short window of time after bacterial inoculation becomes the critical period when the body must prevent bacterial adherence to the implanted hardware. However, the early immune response to infection is dominated by neutrophils, which have been shown to be dysfunctional in diabetic rats. Without an intact innate cellular immune response, *S. aureus* is able to freely colonize the implant and makes the infection untreatable without hardware removal. Absorbance values obtained in this study were normalized due to intra-experimental variability in the absolute absorbance values detected by the spectrophotometer. Because the inoculated and non-inoculated sides for a given animal were analyzed simultaneously, the relative values obtained are believed to be reliable.

**10.4.3: Study limitations**

In this study, limitations exist in drawing conclusions about the infectious susceptibility of our diabetic animals when compared to non-diabetics. Conclusions about infectious susceptibility, as opposed to infectious burden, can be drawn from the bacterial quantification data in the non-inoculated limb. One would assume that the non-inoculated limbs of both groups would have approximately the same degree of bacterial burden if their infectious susceptibility was the same. However, this was not the case, as the diabetic group had increased bacterial burden in the non-inoculated limb when
compared to the non-diabetic group. Even in the absence of direct inoculation, the diabetic group was more susceptible to a more severe post-operative infection than the non-diabetic group. The fact that a significant difference between the two groups could not be detected is most likely due to insufficient power, as the sample size for the study was relatively small. Importantly, the bacterial growth in the non-inoculated limb of both groups was attributed to normal bacterial burden from the operation. Every effort to remain sterile for the entirety of the implant and explant operations was made, and none of the rats were given antibiotics during the perioperative period.

Additionally, the study was limited in its ability to quantify total infectious burden at the surgical site as only the muscle directly overlying the fracture plate was harvested. Although it is unlikely that the infections were localized to two centimeters of tissue, the technique employed provided the accuracy and precision needed to detect statistically significant differences via tissue plate quantification method. Lastly, it is also pertinent to note that the method of directly inoculating the surgical site with concentrated bacteria does not mimic real-life acquisition of a surgical infection. However, this was the best mode available to introduce a known bacterial load into the operative field.

10.4.4: Conclusion
Numerous retrospective clinical studies have looked at diabetic patients’ increased odds of developing an infection after undergoing orthopedic hardware implantation. To date,
no model exists to study this question at the basic science level. The impact of implant infections is substantial with patient morbidity and mortality as well as cost to the health care system needing to be accounted for. To this end, a novel outcomes-based STZ dosing regimen was developed to induce diabetes in 100% of experimental CD rats. The model exceeded the best response rates to STZ reported in the literature and achieved more consistent hyperglycemic values. Additionally, a post-operative *S. aureus* infection in the diabetic rodent model after internal hardware fixation was reliably reproduced. The infectious burden after *S. aureus* inoculation in the presence of implanted hardware is significantly higher in diabetic animals when compared to non-diabetics. These data support the hypothesis that uncontrolled diabetes adversely affects the immune system’s ability to fight and clear infection in the post-operative period.
Part IV: THE USE OF NITRIC OXIDE TO MODULATE THE TISSUE RESPONSE TO INDWELLING IMPLANTS

Chapter 11: Increased in vivo glucose recovery via NO release

*Original article co-authored with BM Klitzman et al.* Adapted with permission from Analytical Chemistry, Increased in vivo glucose recovery via nitric oxide release, 2011, 83(4): 1180-1184, Nichols SP, Le NN, Klitzman BM, Schoenfisch MH. Copyright (2011) American Chemical Society. Text excerpts, and any original copyright notice displayed with material.

11.1: Introduction

Implantable glucose sensors oftentimes fail to function reliably for extended durations (>1 week) primarily due to the foreign body reaction\textsuperscript{122, 123} (FBR). The FBR begins with protein adsorption to the implant and culminates with the formation of a fibrous capsule that sequesters the implant from normal tissue\textsuperscript{124-127}. This isolation results in decreased analyte diffusion and sensor performance\textsuperscript{124, 128, 129}. In addition, infiltration of inflammatory cells during the acute inflammatory response can have a significant effect on the long-term FBR and sensor function\textsuperscript{130}. In order to achieve extended sensor lifetimes, strategies are being developed which mitigate the FBR and improve tissue integration. These strategies, which have had some measure of success in mitigating the FBR, include the use of more hydrophilic interfaces, porous coatings, and the engineering of surfaces that release pro-angiogenic factors and collagen inhibitory agents\textsuperscript{131-133}. 

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Nitric oxide (NO) release is another strategy that may prove beneficial in mediating the FBR and improving analyte diffusion to an implanted sensor. Many of the properties of NO have been mentioned previously, but most importantly, active release of NO from a surface has been shown to reduce bacterial adhesion and infection and to improve implant-tissue integration by reducing inflammatory cell infiltration and collagen encapsulation\textsuperscript{134}. Studies have shown that NO release in the first days of implantation significantly influenced both the short- and long-term inflammatory response\textsuperscript{134, 135}. Therefore, NO release has the potential to address the difficulties of the FBR associated with implantable sensor platforms and to improve sensor function.

One method for testing the effects of NO release on the FBR involves the use of microdialysis, which allows for the direct quantification of glucose diffusion through a membrane. Glucose diffusion and thus sensor performance are certainly affected by the development of a foreign body capsule, and it is likely that a thinner capsule results in enhanced glucose diffusion. Microdialysis probes are calibrated by evaluating the extraction efficiency (EE, equation 1) of a given analyte\textsuperscript{136, 137}. The EE is calculated using the concentration of analyte in the perfusate, dialysate, and external solution represented by $C_p$, $C_d$, and $C_e$, respectively. The resistances to mass transfer through the membrane ($R_m$) and dialysate ($R_d$) are intrinsic to the individual probe and can be
accounted for by in vitro calibration\textsuperscript{136}. The external resistances to mass transfer of biofouling (R\textsubscript{bf}), encapsulation (R\textsubscript{ec}) and tissue trauma layers (R\textsubscript{tr}) are dependent on the host response to the probe once implanted, and may change with time\textsuperscript{137-140}. Glucose consumption may also be increased in wounded tissue and is thus included in the tissue trauma term\textsuperscript{141}.

Equation 1: $$EE = \frac{C_d - C_p}{C_e - C_p} = 1 - \exp\left(-\frac{1}{Q_d (R_m + R_d + R_{bf} + R_{ec} + R_{tr})}\right)$$

Microdialysis probes, similarly to subcutaneous sensors, experience diminished analyte diffusion with longer implantation time\textsuperscript{132, 140, 142}. A previous study conducted by Stenken et al. examined the effect of the FBR on analyte diffusion through a microdialysis membrane using magnetic resonance\textsuperscript{143}. Others have evaluated membrane composition and the active release of vascular endothelial growth factor (VEGF) or dexamethasone to alter analyte recovery\textsuperscript{132, 140, 143}. These strategies were not able to sufficiently mediate the effect of the FBR on analyte diffusion.

A model of \textit{in vivo} glucose recovery involving NO-releasing microdialysis probes implanted subcutaneously in rat subcutis is presented. Saturated NO solutions were used to steadily release NO, resulting in a constant flux of 162 pmol cm\textsuperscript{-2}s\textsuperscript{-1} from the probe membrane over 8 hours of perfusion daily. The \textit{in vivo} effects of enhanced localized NO were evaluated by monitoring glucose recovery over a 14-day period, with
additional histological analysis.

11.2: Materials and methods

11.2.1: Materials

Nitrogen, argon and nitric oxide were purchased from AirGas National Welders (Raleigh, NC). Glucose, glucose oxidase (type VII-S from Aspergillus niger; 168800 units/g), and horseradish peroxidase (type I, 118 units/mg) were purchased from Sigma (St. Louis, MO). CMA/20 microdialysis probes with a 10 mm polyarylethersulfone (PAES) membrane and 20-kDa molecular weight cutoff were purchased from CMA Microdialysis, Inc (North Chelmsford, MA). Bioanalytical Systems (West Lafayette, IN) Baby Bee syringe pumps with 3-syringe brackets, 1 mL Bee Stinger syringes, FEP tubing, PEEK tubing and microdialysis connectors were used to perfuse microdialysis probes. O-dianisidine dihydrochloride was obtained from Alfa Aesar (Ward Hill, MA). All other reagents used were reagent grade and used as received. Phosphate buffered saline (PBS; 10 mM, pH 7.4) was prepared in-house. PBS saturated with NO (PBS-NO; 1.9 mM NO) was prepared by purging approximately 20 mL of PBS with argon gas for 20 min to remove oxygen, followed by nitric oxide gas for 20 min. The solution was stored at 4 °C and used up to 48 h after saturation.

11.2.2: Measurement of NO release

Real-time NO release was collected using a Sievers 280 Chemiluminescent NO Analyzer (Boulder, CO). The instrument was calibrated with an atmospheric sample that had
been passed through an NO zero filter and a 25.6 ppm NO gas standard (balance N₂).

Nitric oxide release from the microdialysis probe was measured by immersing the probe in deoxygenated PBS at 37°C. PBS-NO was then perfused through the probe at 2.0 μL/min with NO carried from the buffer to the NO Analyzer by a stream of N₂ bubbled into the solution at a flow rate of 80 mL/min.

11.2.3: *In vitro* glucose recovery

Prior to implantation, microdialysis probes were sterilized with ethylene oxide (gas treatment), out-gassed for 7 days to facilitate ethylene oxide desorption, and subsequently hydrated in sterile PBS for 24 hours. Probes were then calibrated in a well-stirred solution of sterile 5.5 mM glucose in PBS at a flow rate of 2.0 μL/min with PBS or PBS-NO as the perfusate. Three separate dialysates were collected from each probe and stored at -20 °C until analysis.

11.2.4: Implantation and *in vivo* perfusion of probes

The animal protocol used in this study was approved by an IACUC committee at Duke University. Microdialysis probes were implanted into adult male CD rats (150-200g) purchased from Charles River Laboratories (Raleigh, NC). Rats were anesthetized with 2-4% isoflurane (v/v in O₂). Two probes (one control and one NO-releasing) were then implanted subcutaneously in 10 rats. Probes were placed bilaterally 5-7 cm caudal to the scapulae, approximately 2 cm lateral to the spine, in the dorsal subcutis with the probe tips oriented caudally, and the inflow and outflow percutaneous catheters at the base of
the neck.

PEEK or FEP tubing was used with control probes. PEEK tubing was connected to the inlet of the NO-releasing probes because of its low permeability to NO. Rats were fitted with infusion harnesses with a spring offset attached to a dual-channel stainless steel swivel on a counter-balanced swivel mount (Instech Laboratories, Plymouth Meeting, PA) to allow free range of motion while continuously perfusing probes. Immediately following implantation and for each subsequent day, probes were perfused at 2.0 μL/min for 8 h with test (NO gas) or blank perfusate. During the last 15 min of the perfusion period, one dialysate sample was collected every 5 min and stored at -20° C. Immediately following sample collection, a blood sample was taken via the rat tail vein to allow for blood glucose measurement using a OneTouch Ultra glucose test strip with a OneTouch Ultra Glucometer (LifeScan, Milpitas, CA).

11.2.5: Explantation and fixation of capsules

After 14 days, or after both probes failed, rats were anesthetized with 2-4% isoflurane (v/v in O2), and probes were explanted with the surrounding tissue capsule intact. Microdialysis probes were removed from the capsule if still functional and placed in PBS. The capsules were placed in 10% buffered formalin (v/v) for 24 h and then transferred to 70% ethanol for 24 h prior to their embedment in paraffin. Sections of the paraffin-embedded capsule were stained with Masson's trichrome or hematoxylin and
eosin (H&E) for analysis. Images of the trichrome and H&E stained samples were collected using 4x, 10x and 20x objectives on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS-2Mv Digital Camera (Nikon Inc., Melville, New York).

11.2.6: Glucose detection

All glucose samples were measured using a colorimetric glucose assay in a 96-well microtiter plate format. Phosphate buffer (58.5 μL, pH 7.0) was added to each microtiter plate followed by addition of either dialysate (3 μL) or varying volumes of a standard glucose solution (1-3 μL) for a calibration curve. In the dark, a glucose assay mix (58.5 μL, pH 7.0) containing glucose oxidase (17.2 U/mL), horseradish peroxidase (3.6 U/mL), and o-dianisidine (0.43 mM) was added to each well and incubated at 37° C for 1 h. After incubation, 12N sulfuric acid (80 μL) was added to end the reaction and intensify the color. The absorbance of each well was measured using a Labsystems Multiskan RC microplate reader (Helsinki, Finland) equipped with a 540 nm filter.

11.2.7: Histology

Histological analysis was performed on tissue adjacent to probes functional for ≥ 13 days of implantation. Capsule thickness was measured from trichrome-stained tissue sample. The foreign body capsule was defined as the region of inflammatory cells at the probe surface and the dense collagen oriented parallel to the probe membrane. Two cross-sectional slides per capsule were imaged with seven measurements of the capsule thickness per image and averaged. Collagen density was calculated in four 400 x 100
μm² fields from each of two slides per probe using a previously developed MATLAB (The MathWorks, Natick, MA) program that determines the percent collagen. The inflammatory response as determined by cell density was calculated by counting the nuclei between the dense collagen and probe surface in four 50 x 100 μm² fields from each of two slides per probe using a MATLAB program.

11.3: Results

11.3.1: NO release from microdialysis probes

A NO-saturated PBS solution introduced through the microdialysis probed at 2.0 μL/min resulted in a constant NO flux during the perfusion period (Figure 20). The 8-hour perfusion window enabled the probes to be disconnected, thus reducing mechanical stress on the percutaneous implants. This is important to note as mechanical stress has been shown to affect wound healing in vivo. In PBS, an average NO flux of 162 ± 18 pmol cm⁻²s⁻¹ was measured, which corresponds to a total release of 4.6 ± 0.5 μmol cm⁻² per day. The observed signal from the NO flux was always >3σ greater in magnitude despite constant noise originating from the syringe pump noise (Figure 20).
11.3.2: Glucose recovery: in vitro and in vivo

The extraction efficiency (EE, equation 1) was calculated using either the glucose concentration in the external solution (in vitro) or the blood glucose concentration (in vivo) with the dialysate concentrations. EE values were multiplied by 100 to obtain EE%.

Blood glucose values were used to estimate the glucose concentration in subcutaneous tissue\textsuperscript{146} (Ce). The EE% of glucose for probes in well-stirred in vitro solutions using PBS and PBS-NO was 70±5 and 71±5%, respectively. Because the observed difference was not statistically significant, it allowed for direct comparison between the control and NO-releasing microdialysis probes in vivo. It is important to note that while the in vivo microdialysis flow rate employed (2 μL/min) may deplete local glucose more rapidly than an implanted electrochemical sensor\textsuperscript{141}, the steady-state equilibrium established in vivo at such a flow rate was necessary to quantify the wound healing response. The EE%
of NO-releasing probes remained constant over the 14-day implantation period, while control probes experienced diminished analyte diffusion after 7 days of perfusion (Figure 21).

![Graph showing glucose recovery over time for NO-releasing and control probes.](image)

**Figure 21:** Glucose recovery at various times of implantation for the NO-releasing (red) and control (black) probes. Error bars are ± standard error of the mean. Significant differences (*) are p < 0.05.

### 11.3.3: Histology

Histological analysis of the capsules surrounding NO-releasing and control probes is shown in Figure 22. Control probes had greater fibrous encapsulation compared to NO-releasing probes, as shown in Masson’s trichrome stained tissue cross-sections (Table

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H&E staining (Figure 22) also revealed decreased inflammatory cell densities adjacent to NO-releasing probes relative to controls, which seems to suggest a mitigated FBR. Unexpectedly, the collagen density adjacent to NO-releasing probes was greater than that of control probes.

Figure 22: Representative histology slides of cross sections stained with Masson’s trichrome (A, C) or hematoxylin and eosin (B, D) of NO-releasing (A, B) and control (C, D) probes after 14 days. Arrows in H&E stained pictures indicate the probe membrane. Arrows in the Masson’s trichome stained pictures indicate the implant site, surrounded by dark, stained inflammatory cells, and the collagen capsule. An increased capsule size and inflammatory response at the membrane surface are observed at control probes.
Table 10: Results of histological analysis from both hematoxylin and eosin and Masson’s trichrome stained slides. Results are mean ± SEM.

<table>
<thead>
<tr>
<th>Probe type</th>
<th>Cell density (nuclei / 50 x 100 μm²)</th>
<th>Capsule thickness (μm)</th>
<th>Collagen density (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td>60.4 ± 8.9*</td>
<td>689 ± 61†</td>
<td>62 ± 6*</td>
</tr>
<tr>
<td>NO-releasing (n=4)</td>
<td>37.7 ± 6.6</td>
<td>599 ± 69</td>
<td>72 ± 4</td>
</tr>
</tbody>
</table>

*Significantly different at p<0.05
†Significantly different at p<0.01

11.4: Discussion and conclusions

While many studies have demonstrated the promising anti-infective efficacy and wound healing properties promoted by materials that spontaneously release NO\cite{94, 134, 135}, the long-term benefits of NO on in vivo sensor or probe response have not been determined. Microdialysis allows for the direct quantification of analyte diffusion to an implant surface as a function of implant time. Daily monitoring is possible via perfusate analysis while the tissue remains undisrupted. This considerable advantage can reduce the number of animals required for the study, as well as provide more information than standard tissue histology alone.

To evaluate the effect of NO release during the early stages of the wound healing process, an appropriate method for delivering NO was determined. Initially, controlled NO release from a microdialysis probe was achieved by perfusion of small molecule NO donors. However, the NO release kinetics from these donors was too rapid and resulted
in irregular NO release and bubble formation in the solution. Silica-based nanoparticles were also investigated due to their extended NO release kinetics, however these clogging of these particles in the probe and/or tubing resulted in significantly greater probe failure rates. In addition, microdialysis probe membranes were coated with NO-releasing polyurethane. However this coating considerably inhibited glucose recovery. Steady, controlled NO release was finally achieved using saturated NO solutions.

11.4.1: NO release from microdialysis probes

The measured NO flux was significantly less than expected from the saturated NO solution (1.9 mM NO). This result may be partly due to incomplete diffusion of NO through the probe membrane. In order to reduce NO loss, the inlet tubing was replaced with gas-impermeable PEEK tubing. Unfortunately, the stiffness of the PEEK tubing resulted in a greater rate of probe failure due to significant mechanical stress. Therefore, although the polyurethane resulted in lower (~60%) NO flux, it provided for a functional system, with levels of NO seen in previous studies\textsuperscript{134}.

11.4.2: Glucose recovery: \textit{in vitro} and \textit{in vivo}

Previous studies have shown that fibrous encapsulation inhibits diffusion of small analytes to implant surfaces\textsuperscript{146}. Therefore, one of the results of this study, reduced capsule thickness, suggests improved sensor performance via increased glucose diffusion. Lag in sensor response is generally attributed to greater resistance to mass transfer\textsuperscript{147}. Additionally, decreased analyte diffusion through a highly-resistive fibrous
capsule may affect glucose and oxygen levels at the sensor-tissue interface and inhibit sensor performance\textsuperscript{148}. Localized NO release appears to facilitate glucose diffusion, however, as indicated by the EE%. Therefore, NO-releasing glucose sensor membranes may improve sensor performance over longer implantation periods.

11.4.3: Histology

A study conducted by Wang et al. previously indicated encapsulation of microdialysis probes in a rat model after ~7 days\textsuperscript{139}. It is likely then that the EE% difference between the NO-releasing and control probes results from decreased microdialysis probe encapsulation due to NO release.

The increased collagen density seen in this study is likely due to the effect of NO on the deposition of collagen from fibroblasts. Though a previous study showed a decrease in collagen density for NO-releasing xerogel membranes, others have reported that high concentrations of NO increase collagen deposition in wound healing\textsuperscript{134, 149-151}. The xerogel system exhibited different NO kinetics however, resulting in a large burst of NO initially followed by significantly less (~95%) NO release over approximately 3 days. For this study, NO release levels were equivalent to daily NO bursts with 8-hour durations. The increased collagen density adjacent to NO-releasing probes did not seem to negatively affect glucose recovery however.
Significant migration of the percutaneously-implanted probes within the tissue was observed during the study. This can most likely be attributed to animal movement and the resulting stress from the microdialysis tubing. Mechanical stress would likely augment the FBR to the implanted material. A study by Koshwanez et al. indicated that percutaneous and subcutaneous implants behave differently, possibly due to mechanical stresses\textsuperscript{131}. Future studies might examine the effect of NO as a function of implant type (i.e., subcutaneous vs. percutaneous). Finally, while microdialysis allowed for daily NO release and subsequent analysis of the FBR, probe failure remained a limitation. Total microdialysis probe failure rates were considerable (~30% and ~65% over 8 and 14 days, respectively). A more optimized system would be necessary to study NO release via microdialysis for durations $\geq 14$ d.

11.4.4: Conclusion

This study demonstrated the benefits of \textit{in vivo} NO release with respect to glucose recovery and the FBR using microdialysis. The noticeable difference in EE\% seen in the glucose recovery data suggests improved tissue integration of the microdialysis probe. Additionally, histological analysis indicates that the release of NO reduced both the capsule thickness and inflammatory cell density at the surface. The results support the conclusion that NO release is a viable strategy for mitigating the FBR and for improving analyte diffusion to a sensor.
Chapter 12: The effect of NO surface flux on the foreign body response to subcutaneous implants


12.1: Introduction

Implanted biomedical devices commonly fail due to the events surrounding the foreign body response (FBR). Implantation of these devices disrupts the native tissue, and proteins and other biomolecules immediately adhere to the device surface\textsuperscript{127, 151}. Inflammatory cells then migrate to the wound site and attempt to phagocyte the foreign implant\textsuperscript{127, 151}. Within weeks, a relatively avascular, collagen-rich encapsulation develops around the device, isolating it from the surrounding environment\textsuperscript{126}. Macrophages fuse to form multi-nucleated foreign body giant cells (FBGCs) that enhance the degradation of the implant\textsuperscript{152, 153}. Performance of the device is adversely affected, and in the case of implanted glucose sensors, inaccurate measurements result due to decreased glucose diffusion through the foreign body capsule.

Nitric oxide (NO) releasing coatings have been applied to implantable devices in hopes of mediating the FBR via the roles the molecule plays in cytokine production\textsuperscript{154, 155}, collagen deposition\textsuperscript{150, 156-158}, angiogenesis\textsuperscript{83}, and antimicrobial activity\textsuperscript{159}. A study
conducted by Hetrick et al.\textsuperscript{134} examined the subcutaneous \textit{in vivo} response to NO-releasing \textit{N}-diazeniumdiolated xerogels coated onto rectangular silicone rubber substrates. The xerogels released $\sim 1.35 \, \mu\text{mol}/\text{cm}^2$ of NO over 72 hours with 50\% of the payload exhausted within 5 hours. After 3 weeks of implantation, collagen capsule thickness was reduced by $>50\%$. In addition, NO release decreased the chronic inflammation at both 3 and 6 weeks and improved angiogenesis adjacent to the implant after only 1 week\textsuperscript{134}. Efficacy of NO release was also shown in a study involving NO-releasing microdialysis probes implanted in rat subcutaneous tissue\textsuperscript{160}. Glucose recovery of NO-releasing probes was significantly greater after 7 days of implantation when compared to controls. In addition, NO-releasing probes exhibited thinner collagen capsules and a decreased inflammatory response versus controls\textsuperscript{160}. Finally, in a study conducted by Gifford et al.\textsuperscript{135}, subcutaneous glucose sensors were treated with an NO-releasing polymer matrix. The results showed significant reduction in the \textit{in vivo} inflammatory response, but only when NO was being actively released.

Nitric oxide release from biomaterials has been shown to reduce the FBR, yet the optimal NO release kinetics and doses remain unknown. In a study conducted by Koh et al.\textsuperscript{161}, NO-releasing nanoparticles of various compositions were doped into polyurethane (PU) matrices. The NO release kinetics were then controlled depending on the properties of the PU and/or NO-releasing scaffold. The ability to create a wide
range of NO payloads and release kinetics enables the systematic study of the effect of NO on the FBR from chemically identical interfaces. Thus, a porcine implant model is presented in which polyurethane-coated wire substrates with varying NO release properties were implanted in subcutaneous tissue for 3, 7, 21, and 42 days. Substrates were explanted and adjacent tissue was analyzed via histology.

12.2: Materials and methods

12.2.1: Materials

Tetramethoxysilane (TMOS) and L-proline were purchased from Sigma Aldrich (St. Louis, MO). 3-Mercaptopropyltrimethoxysilane (MPTMS), N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAP3), and tetraethoxysilane (TEOS) were purchased from Gelest (Tullytown, PA). Diethylenetriamine pentaacetic acid (DTPA) was purchased from Fluka (Buchs, Switzerland). Ethanol (EtOH), methanol (MeOH), tetrahydrofuran (THF), and ammonia solution (NH₄OH, 30 wt% in water) were purchased from Fisher Scientific (Fair Lawn, NJ). Stainless steel wire (316L, 381 μm diameter) was purchased from McMaster-Carr (Atlanta, GA). Tecoplast TP-470-000 (TP-470), Tecophilic HP-93A-100 (HP 93A), and Tecoflex SG-80A (TPU) were gifts from Thermedics (Woburn, MA). Hydrothane AL 25-80A (HPU) was a gift from AdvanSource Biomaterials Corporation (Wilmington, MA). Nitric oxide was purchased from Praxair (Danbury, CT). Nitric oxide calibration gas (26.39 ppm; balance nitrogen), nitrogen, and argon were purchased from National Welders (Raleigh, NC). Distilled
water was purified to 18.2 MΩ/cm with a Millipore Milli-Q Gradient A-10 water purification system (Bedford, MA). All other reagents were reagent grade and used as received.

### 12.2.2: Preparation of NO-releasing scaffolds

1-[2-(Carboxylato)pyrrolidin-1-yl]diazen-1-iium-1,2-diolate (PROLI/NO) was prepared by converting the secondary amine in L-proline to an N-diazeniumdiolate following a previously described procedure\textsuperscript{163}. Nitric oxide-releasing silica particles were synthesized based on the sol-gel process via the co-condensation of AEAP3 (70 mol% balance TMOS) or MPTMS (75 mol% balance TEOS)\textsuperscript{164,165}. Subsequent N-diazeniumdiolation of the amine-containing particles was performed under high pressure of NO for 3 days in the presence of sodium methoxide in methanol at room temperature\textsuperscript{164}. Nitrosation of the thiol-containing nanoparticles was carried out by reaction with acidified nitrite in the dark at 0°C\textsuperscript{165}. The details of the NO-releasing characteristics for each system are provided in supporting information (Appendix A, Tables 16-19).

### 12.2.3: Preparation of polyurethane-coated wire substrates

Stainless steel wires were cut to ~5 cm and cleaned by sonicating sequentially in EtOH, water, and EtOH again for 30 min each and sterilized by autoclaving. To create the NO-releasing coating, the NO-releasing vehicle (i.e., PROLI/NO, AEAP3 nanoparticles, or MPTMS nanoparticles) was dispersed into cold EtOH (2.5 mL) at concentrations of 36 or
72 mg/mL. This solution was then mixed with an equal volume of 50:50 wt% HPU/TPU (160 mg/mL total PU) dissolved in THF (2.5 mL) for a resulting concentration of 18 or 36 mg/mL scaffold and 80 mg/mL PU in 50:50 v% EtOH/THF. In a sterile laminar flow hood, wires were then dip-coated four times in the scaffold-containing PU solution with brief ambient drying between dips. A polyurethane topcoat (TP-470, TPU, HPU/TPU, HPU or HP 93A; 40 mg/mL dissolved in THF) was then applied and allowed to dry. The PU-coated wire was then cut to 3 cm, and the freshly-cut end was coated with the same PU topcoat. The PU-modified wires were placed into individual, sterile, microcentrifuge tubes and kept vacuum-sealed in the dark at -20°C until use. Control (non-NO releasing) wires were made using the same protocol, but with no scaffold in the PU solutions.

12.2.4: Characterization of polyurethane-coated wire substrates

Scanning electron microscopy was used to evaluate the macroscopic surface roughness of the coated wires using a Quanta 200 (FEI, Hillsboro, OR) in high-vacuum mode before and after the top-coating process. Release of NO was measured continuously using a Sievers 280i Chemiluminescence Nitric Oxide Analyzer (NOA) (Boulder, CO) \(^{166}\). Calibration of the NOA was performed with both air passed through a Sievers NO zero filter and 26.39 ppm NO gas (balance N\(_2\)). For analysis, NO-releasing wire substrates were immersed in 25 mL of deoxygenated phosphate buffered saline (PBS; 10 mM, pH 7.4). Released NO was carried to the analyzer in a nitrogen stream (200 mL min\(^{-1}\)).
Temperature control was maintained using a water bath at 37°C. Nitric oxide release from nitrosothiol nanoparticle-doped coatings were studied by shielding the sample flask from light and using PBS with 500 μM DTPA to chelate trace copper. The thickness of the wire coatings was estimated by optical microscopy.

12.2.5: Silicon elemental analysis
To characterize particle stability (via leaching) in the various polyurethane polymers, substrates were incubated at 37°C in PBS (1 mL) for 1, 3 and 6 weeks. Samples were then further prepared for Si elemental analysis by adding aqua regia (2.5 mL), hydrofluoric acid (1 mL), and 40% triethanolamine (3.575 mL) to dissolve the particles, and then diluted to 50 mL with water. To determine the mass of silica nanoparticles contained in the PU films, the coatings were dissolved in piranha solution (1 mL), exposed to hydrofluoric acid (1 mL) and 40% triethanolamine (3.575 mL), then diluted to 50 mL with water. (CAUTION: hydrofluoric acid and piranha are extremely corrosive and require special handling). The silicon concentration in the solutions was subsequently measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES; Prodigy, Teledyne Leeman Labs, Hudson, NH).

12.2.6: Implantation and explantation of wire substrates
The animal protocol used in this study was reviewed and approved by the IACUC at Duke University. Coated wire substrates were implanted into seventeen mixed breed Yorkshire-type piglets weighing approximately 5–7 kg. Pigs were initially anesthetized
with ketamine:xylazine (20 mg/kg and 2 mg/kg, respectively) and maintained on 2–4% isoflurane (v/v in O₂) during implantation. The dorsal skin was prepared by clipping of the hair and triplicate scrubbing with chlorhexidine and alcohol. Four 1-cm incisions were created 4 cm lateral to the dorsal midline and 8 cm and 18 cm caudal to the scapulae using a scalpel. Five or six wires were then inserted radially (“clock hour” pattern) in the 2, 4, 6, 8, 10, and 12 o’clock positions, extending 2 cm out from the incision. The 6 o’clock position was eliminated in two caudal implant sites, resulting in 22 wires implanted per pig.

After 3, 7, 21, or 42 days, pigs were anesthetized and the tissue surrounding the wire implants was explanted and placed into 10% buffered formalin (v/v) for 24 h, then transferred to 70% EtOH (v/v in H₂O) for at least 24 h prior to embedding in paraffin. Sections of the paraffin-embedded tissue were stained with Masson’s trichrome or hematoxylin and eosin (H&E). Micrographs of the trichrome and H&E stained samples were collected using 4, 10, and 20x objectives on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS 2Mv digital camera (Nikon Inc., Melville, NY).

12.2.7: Histology

Histological analysis was performed on all explanted tissue samples. Capsule thickness was measured from Masson’s trichrome-stained tissue sections. The foreign body capsule was defined as beginning at the edge of the implant and consisting of the region
of dense collagen oriented parallel to the implant. The end of the capsule thickness was
determined to be where collagen was no longer the primary tissue constituent (indicated
by a decrease in the density of blue—green stain) nor oriented parallel to the surface.
Eight capsule thickness measurements taken radially at 45° intervals were averaged for
each image. Three images of the collagen capsule were processed using a previously
developed MATLAB (The MathWorks, Natick, MA) program that quantifies the percent
collagen. The program defines collagen from trichrome-stained images by the
characteristic blue—green color, divides the number of collagen positive pixels by the
total pixels in the image, and multiplies this ratio by 100 to obtain percent collagen.
Hematoxylin and eosin-stained tissue sections were used to measure the inflammatory
response. The inflammatory response, as determined by cell density, was determined
by counting all cell nuclei within 50 μm of the implant surface from three 50 x 100 μm²
fields from each slide. Cell nuclei were defined as the purple-stained, spherical features
in the H&E-stained tissue sections. Histology data are expressed as mean ± standard
error of the mean and tested for significance (i.e., p < 0.05) using a non-parametric
Wilcoxon rank-sum test.

12.3: Results

12.3.1: Characterization of polyurethane coatings

Wire substrates were coated with particle-doped polyurethanes (PUs) capable of NO
release. The thickness of the PU coatings remained constant regardless of the
incorporation of a range of NO-releasing scaffolds and concentrations. For instance, control PU and 36 mg/mL MPTMS nanoparticle-doped PU coatings were 46 ± 4 µm and 49 ± 4 µm, respectively. Initially, particle-doped films exhibited markedly greater surface roughness than controls as observed using SEM (Figure 23A). Therefore, a PU topcoat was added to all coatings to ensure that any tissue response observed in vivo was a result of NO release and not surface topography (Figure 23B).

![Figure 23](image)

**Figure 23**: Scanning electron microscope images of polyurethane-coated wire substrates dipcoated four times in a polymer solution mixture of 36 mg/mL MPTMS nanoparticles and 80 mg/mL HPU/TPU A) before, and B) after topcoating with a 40 mg/mL HPU/TPU solution. Nanoparticle-induced surface roughness is masked after topcoating.

Leaching of nanoparticles was quantified to address cytotoxicity concerns although silica is generally considered non-toxic\(^{167}\). The mass of nanoparticles within the coatings was measured by dissolving the PU coatings and quantifying via ICP-OES. Wires dipcoated in 36 mg/mL AEAP3 nanoparticle-doped PU and 36 mg/mL MPTMS nanoparticle-doped PU contained 1.11 ± 0.19 mg and 1.24 ± 0.13 mg of silica nanoparticles,
respectively. Over 6 weeks, MPTMS soak solutions had no detectable leaching. The AEAP3 nanoparticles however, leached $4.2 \pm 0.7\%$ of the total loaded scaffold, with the majority of leaching (>90%) occurring during the first 3 weeks. This greater degree of leaching can likely be attributed to particle size (AEAP3 ~110-150 nm; MPTMS ~750-900 nm).

**12.3.2: Nitric oxide release from polyurethane coatings**

Several NO-releasing scaffolds were employed to evaluate the effect of NO release kinetics on the FBR, including PROLI/NO, AEAP3 nanoparticles, and MPTMS nanoparticles. The low molecular weight (LMW) N-diazeniumdiolate PROLI/NO provided the fastest NO release with >90% of the NO payload liberated after 6 min. Longer durations of NO release from N-diazeniumdiolate donors were achieved using a hybrid silica nanoparticle scaffold containing AEAP3. NO release kinetics were further tuned for N-diazeniumdiolate systems by selection of the PU topcoat. The hydrophobicity of the topcoat dictates water uptake through the coating and thus the release of NO, which is proton-initiated in these systems$^{168, 169}$. These topcoats used are listed in Table 11 and were chosen in part due to their water uptake$^{161}$. The longest NO release kinetics were obtained using MPTMS nanoparticles. Complete NO release data for the different scaffolds can be viewed in Appendix A.
Table 11: Water uptake of polyurethane topcoats

<table>
<thead>
<tr>
<th>Polyurethane</th>
<th>Water uptake (mg H₂O/mg PU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tecophilic HP-93A-100 (HP 93A)</td>
<td>2.56 ± 0.31</td>
</tr>
<tr>
<td>Hydrothane AL 25-80A (HPU)</td>
<td>0.63 ± 0.34</td>
</tr>
<tr>
<td>Tecoflex SG-80A (TPU)</td>
<td>0.20 ± 0.18</td>
</tr>
<tr>
<td>Tecoplast TP-470-000 (TP-470)</td>
<td>0.04 ± 0.05</td>
</tr>
</tbody>
</table>

12.3.3: Collagen deposition

Expectedly, collagen capsule formation adjacent to PU-coated wire substrates was not observed until 3 weeks following implantation. Collagen capsule thickness values for all wire substrates at 3 and 6 weeks of implantation are summarized in Figure 24. After 3 weeks, PROLI/NO-doped PUs showed no significant reduction in the collagen capsule thickness relative to control (Figure 24A). However, both the TPU and TP-470 PU topcoats resulted in significantly reduced collagen capsule thickness for both concentrations of AEAP3 nanoparticle-doped PUs versus controls. These hydrophobic topcoats allowed for the longest NO release possible for the N-diazeniumdiolate particle-doped PU systems. Longer NO-releasing MPTMS nanoparticle-doped PUs also significantly reduced collagen capsule formation at 3 weeks.
Figure 24: Collagen capsule thickness surrounding polyurethane-coated wire substrates at A) 3 and B) 6 weeks. Significant differences between NO-releasing and relative controls are indicated at p < 0.05 (*). At 6 weeks, the TP-470 topcoated 18 mg/mL AEAP3 nanoparticle system was not tested for statistical significance due to low sample size (n = 2).

At 6 weeks, the average collagen capsule thickness was reduced for all implanted materials compared to at 3 weeks. A similar decrease in collagen encapsulation over time has been observed for non-NO-releasing materials\textsuperscript{173}. PROLI/NO-doped PUs did not significantly reduce the collagen capsule thickness at 6 weeks (Figure 24B). Of the 18 mg/mL AEAP3 nanoparticle-doped PUs, only the HPU/TPU topcoated system displayed a significant reduction in capsule thickness (∼ 59% reduction vs. control) at 6 weeks. Greater concentrations of NO donor proved effective, as the three longest NO-releasing PU topcoats (i.e., HPU/TPU, TPU, and TP-470) applied to the 36 mg/mL AEAP3 nanoparticle-doped PU systems were capable of significant reduction in the capsule thickness at 6 weeks. Finally, both concentrations of the MPTMS nanoparticle-doped
polyurethanes significantly reduced capsule thickness at 6 weeks with the 18 and 36 mg/mL MPTMS nanoparticle systems achieving a ~ 50 and ~ 76% reduction in capsule thickness, respectively.

Collagen density values for all implants at 3 and 6 weeks are presented in Figure 25. A previously developed and implemented MATLAB program was used to quantify collagen density in captured micrographs of the collagen capsule. The program measures the number of pixels attributed to collagen when stained with Masson’s trichrome, divides this number by the total pixels in the image, and multiplies by 100 to give a collagen density index (CDI) ranging from 0 to 100, with a CDI value of 100 indicating every pixel represents collagen. At 3 weeks, the capsules surrounding the HP 93A, HPU, and TPU-topcoated PROLI/NO systems exhibited significantly increased CDI compared to controls (Figure 25A) with no other NO-releasing substrates exhibiting a significant change. At 6 weeks, only the capsules surrounding the TP-470 topcoated 36 mg/mL AEAP3 and HPU/TPU topcoated 36 mg/mL MPTMS nanoparticle systems showed significantly increased CDI versus controls (Figure 25B).
Figure 25: Collagen density index (CDI) of collagen capsules surrounding polyurethane-coated wire substrates at A) 3 and B) 6 weeks. Significant differences between NO-releasing and relative controls are indicated at $p < 0.05$ (*). At 6 weeks, the TP-470 topcoated 18 mg/mL AEAP3 nanoparticle system was not tested for statistical significance due to low sample size ($n = 2$).

12.3.4: Inflammatory response

Figure 26 shows the inflammatory response as measured by inflammatory cell density of all coated substrates at 3, 7, 21, and 42 days. During the acute phase (3 and 7 days), NO release from a few substrates significantly reduced the inflammatory response (Figure 26A and B). The inflammatory response was most significantly impacted by substrates with a great NO payload and long NO release (e.g., 36 mg/mL AEAP3 and MPTMS nanoparticle systems). The PROLI/NO and 18 mg/mL AEAP3 nanoparticle systems, with NO durations up to 48 h, did not decrease the inflammatory response at 3 or 7 days. Interestingly, the HPU/TPU topcoated 18 mg/mL AEAP3 nanoparticle system was observed to decrease the inflammatory response at 3 days, likely due to the achievement
of optimal release kinetics and duration via the HPU/TPU topcoat. Of the 36 mg/mL AEAP3 nanoparticle systems, the three topcoats with the slowest NO release kinetics (i.e., HPU/TPU, TPU, and TP-470) also significantly reduced the inflammatory cell density adjacent to the implant at 3 days. At 1 week, only the 36 mg/mL MPTMS nanoparticle system significantly reduced the inflammatory response. The chronic inflammatory response (3 and 6 weeks) was largely unaffected by NO release as no significant reduction in inflammatory response was observed for any NO formulation (Figure 26C and D).
Figure 26: Inflammatory response to polyurethane-coated wire substrates at A) 3 d and B) 1, C) 3, and D) 6 weeks. Significant differences between NO-releasing and relative controls are indicated at \( p < 0.05 \) (*). At 6 weeks, the TP-470-topcoated 18 mg/mL AEAP3 nanoparticle system was not tested for statistical significance due to low sample size (\( n = 2 \)).
12.4: Discussion and conclusion

12.4.1: Characterization of polyurethane coatings

Particle-doped polyurethanes (PUs) capable of releasing NO were successfully dip-coated onto wire substrates. Wire substrates were used in order to mimic the shape and size of a standard electrochemical glucose sensor. Properties such as size and shape have both been shown to affect the FBR [37,38]. Additionally, previous in vivo studies indicate that surface roughness may significantly alter the FBR [39, 40]. For this reason, a PU topcoat was added (Figure 1B) to all coatings to ensure that the tissue response observed in vivo was a result of NO release and not physical surface properties.

12.4.2: Nitric oxide release from polyurethane coatings

Both N-diazeniumdiolate and S-nitrosothiol NO donors exhibit pseudo first-order release kinetics, resulting in an initial maximum and then exponential decay in NO flux. Because of rapid NO donor breakdown, LMW PROLI/NO-doped PUs released NO rapidly (~24 h) and exhibited the greatest NO fluxes (1436-3128 pmol cm$^2$s$^{-1}$) (Appendix A). Large bursts of NO may adversely affect the FBR however, as some studies have shown that large amounts of exogenous NO inhibit platelet adhesion and aggregation and slow early healing$^{170, 171}$. The NO release from AEAP3 nanoparticle-doped PUs was significantly longer in duration (~72 h) due to the longer half-life of the AEAP3 nanoparticles (Appendix A). The total NO payload for any given scaffold and
concentration for the PROLI/NO and AEAP3 systems was the same regardless of PU topcoat.

NO release from S-nitrosothiols is not proton-initiated, but rather decomposition occurs in the presence of heat, light, or Cu²⁺. In this case, 1 mol of NO is formed per mol of thiol. Since decomposition is not affected by water uptake, the type of polyurethane used as a topcoat is irrelevant. Therefore, only the HPU/TPU polyurethane topcoat was utilized for MPTMS nanoparticle-doped PUs. The S-nitrosothiol nanoparticles released lower levels of NO over longer periods (~14 days) in comparison to the N-diazeniumdiolate systems (Appendix A). Previous studies examined materials capable of uninterrupted NO release for up to 3 days. The 36 mg/mL MPTMS nanoparticle-doped PUs produced the highest NO payload (9.3 μmol cm⁻²) in this study, an amount ~7 times greater than previous subcutaneous NO-releasing xerogels.

Slight loss of NO occurred during the study as the predicted total NO release for the 36 mg/mL AEAP3 and MPTMS nanoparticle-doped PUs (based on the nanoparticle concentrations measured via the ICP-OES leaching study) was 6.3 and 10.1 μmol cm⁻², respectively. This loss most likely occurred during the wire dip-coating process when exposure to moisture, light, and heat are difficult to control. The range of NO release
kinetics utilized in this study allowed for the investigation of the effects of NO release flux (initial burst versus sustained delivery) to be compared.

**12.4.3: Collagen deposition**

Collagen encapsulation is a characteristic event of the FBR that typically begins to form 1-2 weeks after implantation. The avascular capsule generally persists for the lifetime of the device, impeding diffusion of analytes from the surrounding tissue and microvessels. In the case of glucose sensors, the collagen capsule results in reduced sensitivity and greater lag time. Capsule thickness and capsule collagen density was thus evaluated as a function of NO release kinetics and doses.

Collagen capsule thickness at 3 weeks was significantly reduced by NO-releasing systems with greater NO payloads and longer release durations. The data from the AEAP3 nanoparticle-doped PUs suggest that longer NO-releasing substrates with similar NO payloads more successfully mitigate the FBR. Additionally, the total NO payload may be less important than the NO release kinetics for reducing collagen encapsulation at 3 weeks. For instance, the TP-470-topcoated 18 mg/mL AEAP3 nanoparticle-doped PU has a lower total NO payload but a greater NO flux at 48 h than HP 93A, HPU or HPU/TPU topcoated 36 mg/mL AEAP3 nanoparticle-doped PUs (Appendix A). These slower NO release kinetics correlate well with reduced collagen capsule thickness. The even greater duration NO-releasing PUs (i.e., MPTMS
nanoparticle-doped) significantly reduced the collagen capsule at both concentrations of 18 and 36 mg/mL by ~64 and ~77%, respectively. Indeed, the MPTMS nanoparticle-doped PU systems represented the two largest decreases in collagen capsule thickness at 3 weeks.

Of the 18 mg/mL AEAP3 nanoparticle-doped PUs, only the HPU/TPU topcoated system displayed a significant reduction in capsule thickness (~59% reduction vs. control) at 6 weeks. The other 18 mg/mL AEAP3 nanoparticle-doped PU systems with slightly longer or shorter NO durations proved inadequate in altering the capsule thickness. It is possible that the HPU/TPU topcoat resulted in both a sufficiently high maximum NO release and duration to affect capsule formation. Other PU topcoats may have resulted in kinetics that were sub-optimal. However other relevant parameters likely play a role, as materials with a greater maximum flux and similar NO flux at 48 h (e.g., HPU-topcoated 36 mg/mL AEAP3 nanoparticle-doped PUs) did not result in similar reductions in capsule thickness. The higher incidence of significant capsule thickness reductions with the 36 versus the 18 mg/mL AEAP3 nanoparticle system suggests a possible advantage to materials releasing a greater NO payload. The more hydrophobic PU topcoats (i.e., HPU/TPU, TPU, and TP-470) provided the longest NO release duration for the 36 mg/mL AEAP3 nanoparticle system albeit with identical NO payloads to the statistically insignificant PU topcoats (i.e., HP 93A and HPU). While both MPTMS
nanoparticle systems significantly reduced collagen capsule thickness at 3 and 6 weeks, the greater concentration was able to produce a greater reduction. Since the long-term NO release fluxes (i.e., 7–14 d) for both the 18 and 36 mg/mL MPTMS nanoparticle systems were similar (Appendix A), the difference in the initial 7 d of NO release is attributed to the enhanced FBR mitigation. The combination of significant NO levels initially and sustained, low NO release over 14 days may improve tissue integration by altering the initial inflammatory response as reported by Hetrick et al.\textsuperscript{134}. The 36 mg/mL MPTMS nanoparticle-doped systems reduced the collagen encapsulation to the greatest level at both 3 and 6 weeks of implantation, indicating the advantage of a large initial NO payload and sustained NO release for decreasing capsule thickness.

The density of the collagen within the encapsulation is another factor that may affect implant survival and performance. At 3 weeks, PROLI/NO systems, which exhibit an initial burst release of NO, showed significantly greater collagen density in comparison to controls. Of note, the maximum NO flux from PROLI/NO-doped PUs was an order of magnitude greater (1436-3128 pmol cm\(^{-2}\) s\(^{-1}\)) than that from any previously implanted NO-releasing materials. Though the total NO payload (∼4 μmol/cm\(^2\)) from the PROLI/NO systems falls between that of the two concentrations of AEAP3 nanoparticle systems, neither concentration of AEAP3 nanoparticle systems resulted in significantly increased CDI at 3 weeks. Therefore, the CDI enhancement is most likely the result of
the initial NO bolus from the PROLI/NO systems. The inability to significantly reduce capsule thickness while likewise causing an increase in the collagen density suggests long-term disadvantages of bolus NO release from implant surfaces.

At 6 weeks, NO formulations with the greatest NO payloads and longest release durations resulted in significantly greater CDI in comparison to controls. This enhancement of collagen density at 6 weeks may also be the result of excessive NO. In a previous study involving microdialysis probes\textsuperscript{160}, high NO payloads (4.6 μmol cm\textsuperscript{-2} each day) also resulted in increased collagen density. Numerous studies have indicated that NO stimulates collagen deposition from fibroblasts\textsuperscript{150, 157, 158}. Despite increased CDI however, the NO-releasing microdialysis probes exhibited significantly greater glucose recovery compared to control probes\textsuperscript{160}. While the TP-470-topcoated 36 mg/mL AEAP3 and the HPU/TPU-topcoated 36 mg/mL MPTMS nanoparticle systems were characterized by increased CDI, both of these materials significantly reduced the thickness of the collagen encapsulation. Therefore, the materials may still be advantageous for certain subcutaneous device applications (e.g., glucose sensors).

12.4.4: Inflammatory response

Migration of inflammatory cells to the implant site may contribute to erratic device (e.g., sensor) performance and eventual device failure\textsuperscript{148}. Nearby inflammatory cells become activated while attempting to phagocytose the foreign body. This process decreases
local pH and results in the production of superoxide and peroxide, which have been linked to poor sensor performance\textsuperscript{148, 175, 176}. Over time, the formation of foreign body giant cells (FBGCs) also enhances implant degradation\textsuperscript{152, 153}. The inflammatory response was evaluated by quantifying the number of cell nuclei localized within 50 μm of the implant surface in hematoxylin and eosin-stained histology sections. The inflammatory response to the NO-releasing substrates was examined histologically at both acute (3 and 7 d) and chronic (3 and 6 week) stages.

NO release was able to significantly reduce the inflammatory response in the acute phase. In general, NO formulations with the greatest payloads and longest release durations were effective at mediating the FBR. Only one formulation, the 36 mg/mL MPTMS nanoparticle system, significantly reduced inflammatory cell density at 1 week. The chronic inflammatory response, occurring after all NO payloads were exhausted, was unaffected by NO release as no NO formulation significantly affected inflammatory cell density at later timepoints. At 6 weeks, the 36 mg/mL MPTMS nanoparticle system produced the greatest response, an apparent 22% reduction in the inflammatory response ($0.05 < p < 0.10$). A study conducted by Gifford et al. indicated that the inflammatory response only seemed to be reduced while NO was actively released\textsuperscript{135}. The results of this study are in good agreement with the Gifford et al. study, but are contradictory to a previous study in which a short-term (72h) NO-releasing xerogel
system significantly improved the chronic inflammatory response\textsuperscript{134}. One possible explanation for these results is that these previous studies were conducted in rodent models that may differ in immune response to porcine models\textsuperscript{134, 135, 162}. Nevertheless, the results of this study emphasize the need to develop NO formulations capable of longer release durations, as these seem most effective at mitigating the FBR.

\textbf{12.4.5: Conclusion}

The mitigation of the FBR observed in this study may prove beneficial for indwelling glucose sensors. The results highlight the need to examine NO release kinetics in the development of implantable materials. In particularly, there is a need to create materials with NO release durations exceeding two weeks, as this appears to be relevant in mitigating the inflammatory response. Possible approaches for enhancing NO release durations include increasing the hydrophobicity of the polymer matrix or employing longer releasing S-nitrosothiol NO donors (e.g., tertiary S-nitrosothiols). These modifications however may affect glucose sensor response and performance.
Part V: THE TISSUE RESPONSE TO PERCUTANEOUS FUNCTIONING GLUCOSE SENSORS IN HEALTHY AND DIABETIC ANIMALS

Chapter 13: Comparison of the tissue response to percutaneously implanted glucose sensors in healthy and diabetic animals

13.1: Introduction

This study investigated the tissue response to percutaneously-implanted glucose sensors in healthy and diabetic rats. A multi-dose regimen of streptozotocin was used to induce diabetes in experimental rats. Three types of implantable glucose sensors, supplied by Medtronic® were used: Sof™ sensor, Enlite™ sensor, and Enlite 2™ sensor. Enlite™ and Enlite 2™ sensors represent newer glucose sensor designs intended to promote a less aggressive foreign body response. The designs of these sensors are thinner and more flexible. The Enlite 2™ sensor differs from the Enlite™ sensor in that it contains only the flexible electrode with no outer tubing. At 3 days, 1 week, and 4 weeks, tissue directly adjacent to the sensors was evaluated for collagen encapsulation, density of any collagen encapsulation, inflammatory response as measured via inflammatory cell density, and microvessel density. These endpoints were evaluated histologically via Masson’s trichrome, Hoechst, H&E, CD31, and CD68 staining. Additionally, continuous functional sensor data was evaluated for sensor performance, accuracy, and lag time.
13.2: Materials and methods

13.2.1: Materials

Glucose sensors (Sof™, Enlite™, Enlite 2™) were provided by Medtronic MiniMed (Northridge, CA). 50% dextrose solution was purchased from Nova-Tech, Inc. (Grand Island, NE). Humalog® rapid-acting insulin (Eli Lilly & Company, Indianapolis, IN) was purchased from Duke University Hospital Pharmacy. Aviva Accu Check glucometer and glucose strips were obtained from Roche Diagnostics (Indianapolis, IN). Vetrap 3M animal wrap was obtained from Animal Care Products (St. Paul, MN).

Masson’s trichrome and H&E staining kits were purchased from Sigma-Aldrich (St. Louis, MO). Acetone and glacial acetic acid were obtained from VWR International (Radnor, PA). Normal donkey serum and Alexa Fluor® 488-conjugated AffiniPure Donkey Anti Mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Purified NA/LE Mouse Anti-Rat CD31 was purchased from BD Biosciences (San Jose, CA). Hoechst 33342 was purchased from Sigma-Aldrich (St. Louis, MO).

13.2.2: In vivo studies

All National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. Approval for these studies was granted by the Duke University Institutional Animal Care and Use Committee prior to initiation of the studies. This protocol was approved by the Duke University IACUC.
Male Sprague-Dawley (CD) rats (150-200g) were obtained from Charles River Laboratories (Raleigh, NC). Rats were divided into two experimental groups, non-diabetic and diabetic. Diabetes was induced in the experimental group of animals via daily intraperitoneal (IP) injections of STZ at 40 mg/kg for 3 consecutive days. Following STZ injections, rats were immediately given drinking water supplemented with sucrose or glucose (15 g/l) for 48h to limit early mortality. STZ treatment results in destruction of the pancreatic beta cells and the release of large amounts of insulin; severe hypoglycemia is possible without supplemented drinking water. Approximately 48 hours after the third injection, 3-hour fasting blood glucose measurements were taken from the tail vein of the rats using a standard Aviva Accu Check glucometer (Roche Diagnostics, Indianapolis, IN). Fasting measurements were taken to control for inter-animal feeding variability. Animals were considered diabetic when their fasting blood glucose exceeded 300 mg/dl (16 mM). However, all animals with a blood glucose level less than 350 mg/dl received a fourth dose of STZ. This was done to ensure that animals that were borderline diabetic (blood glucose 300-350 mg/dl) did not subsequently drop below our diabetic threshold over the course of the experiment. Occasionally, blood glucose soon after STZ treatment can be above the diabetic target of 300 mg/dl, but some recovery of insulin production can lead to a small drop below the minimum target range. Approximately 48 hours later we repeated the fasting blood glucose measurements and injected animals with a fifth dose of STZ if their blood glucose was
less than 350 mg/dl. This injection sequence was repeated until all animals in the experimental group reached target blood glucose range of 350-600 mg/dl. All animals were given food and water *ad libitum* and kept on a 12-hour light/dark cycle.

**13.2.3: Anesthesia**

Rats were anaesthetized with 2.5% isoflurane (Baxter Healthcare Corp., Deerfield, IL) in oxygen at a flow of 1 l/min, which was adjusted to effect. Puralube® ocular lubricant (Pharmaderm, Melville, NY) was applied to each rat eye to prevent corneal drying. Rats were also kept on a water-based heating pad to prevent hypothermia during surgery.

**13.2.4: Implantation of glucose sensors**

Animals were implanted with glucose sensors approximately 3 weeks from the time of receipt. Diabetic animals were implanted fourteen days after developing diabetes, a process that takes about one week. Animals were brought into the preparatory room, and anesthesia was induced with 2% isoflurane in oxygen. Once a surgical plane of anesthesia was established, the entire back of the animals were shaved, and the animals were pre-scrubbed with alcohol pads. The animals were then transferred to the operating room and placed prone on the operating table. A rectal thermometer was inserted and the intra-operative temperature of the animals was monitored for the entirety of the operation. The entire back was prepared for surgery first with three treatments of chlorhexidine gluconate soaked sterile gauze followed by three treatments of alcohol pads. The animals were then draped with sterile towels and the back was
allowed to dry before puncture wounds were made. Typically, one Sof™ and one Enlite™ sensor was implanted in each rat, however in a few cases, more than two sensors were implanted in a rat. In a smaller subset of animals, Enlite 2™ sensors were implanted. The implantation procedure for the Enlite™ and Enlite 2™ sensors was identical. All sensors were singly wrapped in sterile packaging. For the Enlite™ sensor, an 18-gauge needle was used to create an entry point into the subcutaneous space approximately 2 cm below the scapulae on the midline. The plastic film covering the adhesive portion of the Enlite™ sensor was carefully removed and the adhesive was trimmed slightly. Great care was taken during this process so as to not accidentally activate the spring-loaded mechanism that retracts the introducer needle from the sensor. A few drops of super glue were applied to the adhesive directly underneath the plastic hub, taking care not to get super glue on the sensor. The introducer needle and sensor were then carefully inserted through the entry point made by the 18-gauge needle, and the loose skin was pulled up while the sensor was pressed into the skin. This procedure was to ensure that the sensor was fully within the subcutaneous space and that the sensor hub was completely flush with the skin. Once the sensor hub was well adhered to the skin, 3-0 or 4-0 nylon monofilament suture was used to attach the hub to the skin via the two tying platforms on the hub (Figure 27). Once the sutures were in place (one suture on each side of the plastic housing), the spring-loaded mechanism was activated, retracting the introducer needle and releasing the sensor into
the subcutaneous space. For the Sof™ sensor, a 12-gauge needle was used to make an entry point and subcutaneous path approximately 6-8 cm below the entry point for the Enlite™ sensor. The plastic film covering the adhesive portion of the Sof™ sensor was carefully removed and super glue was applied to the underside of the plastic housing. The introducer needle and sensor were then carefully inserted through the entry point until the sensor was completely within the subcutaneous space. The sensor hub was then pressed firmly to the skin. Once the sensor hub was well adhered, 3-0 or 4-0 nylon monofilament suture was used to attach the hub to the skin via the two tying platforms on the hub. The introducer needle was then manually retracted, leaving the sensor within the subcutaneous space (Figure 27). Additionally, as available, nonfunctional sensors, which consisted of functional Sof™ and Enlite™ removed from their plastic housing, were fully implanted in the subcutaneous space. These nonfunctional sensors were utilized to determine if micromotion has an adverse effect on the wound healing adjacent to implanted devices.
Figure 27: Implantation procedure for Medtronic® glucose sensors. A) Spring-loaded introducer needle and Enlite™ sensor. B) 12-gauge needle used to make an entry point for the Sof™ sensor. C) Image of sensor locations on rat dorsum. Sutures can be seen on tying platforms.

13.2.5: Attachment of transmitters and animal wrapping

Once the sensors were implanted, the sterile drapes were removed and iPro transmitters with initialization were prepared for connection to the sensors. iPro transmitters were fully charged, cleaned, and time-stamped if necessary. (Previously, GST transmitters were used for in vivo experiments, however, considerable noise in the data made analysis difficult). One iPro transmitter was attached to each sensor (Figure 28). Once attached, the transmitter will display a green blinking dot if it is connected to a functional, fully-hydrated sensor. The transmitter serial number, sensor type, and rat identity were recorded. After transmitters were attached, the rat was wrapped in two layers with Vetrap 3M animal wrap (St. Paul, MN) (Figure 31). Interrupted sutures (2-0
or 3-0) were used to secure the edges of the wrap. The wrapping was snug to prevent the animal from chewing loose portions and to secure the external hardware, but not too tight as to avoid problems with respiration or restriction of movement.

Figure 28: Transmitters attached to implanted functional sensors (top) and Vetrap 3M rat jacket (bottom).
After the Vetrap 3M jacket was in place, animals were allowed to breathe 100% oxygen until spontaneous movement was detected. The animals were then returned to their cage and monitored until sternal recumbency was maintained before being returned to the Vivarium. Rats were housed singly and allowed access to food and water *ad libitum.*

Rats were monitored daily for signs of jacket wear, transmitter attachment/stability, or sensor extrusion. In cases of jacket wear, rats were taken to the OR, re-anesthetized, and the jacket was replaced.

**13.2.6: Sensor calibration**

Sensors were calibrated by taking a tail vein blood measurement using a 27-gauge needle and a standard Aviva Accu Check glucometer and glucose strips (Indianapolis, IN). Sensors were calibrated 2 and 8 hours after the initial connection to the transmitter on the day of implantation. Afterwards, sensors were calibrated at least twice a day with measurements taken no greater than 12 hours apart.

**13.2.7: Insulin and glucose challenges**

One day after sensor implantation and then once a week (for one month time points), rats were administered insulin and glucose challenges that were designed to test sensor performance and lag time. A blood glucose measurement was first taken before the challenges to establish a baseline blood glucose level. Animals were then anesthetized with 2% isoflurane in oxygen and a 25-gauge butterfly needle attached to a 5-ml syringe
containing saline was inserted into the tail vein. Infusions via tail vein were chosen over intraperitoneal (IP) injections because changes in blood glucose occur much more rapidly. Faster changes in blood glucose levels allows for more precision in calculating lag time and evaluating sensor performance as well as decreasing the amount of time necessary to complete an insulin/glucose challenge. (In cases where the vein was difficult to find, boluses were administered via IP injection.) Once the needle was in the vein (confirmed by ability to draw blood into syringe), the syringe containing saline was replaced with a syringe containing Humalog® rapid-acting insulin (10U/ml). Humalog® was chosen due to its rapid action; the drug has a peak time of 10-20 minutes and clears from the body within 2-3 hours. Insulin was then infused slowly into the vein. The syringe containing insulin was then replaced with the syringe containing saline, and the tubing was flushed with saline to ensure all insulin was infused. Non-diabetic rats received an insulin dosage of 2.5 U/kg, while diabetic rats received an insulin dosage of 20.0 U/kg. After infusion, the animals were removed from anesthesia and allowed to wake up. Blood glucose measurements were taken using an Aviva Accu Check glucometer (Indianapolis, IN) approximately every 15 minutes until control rats reached blood glucose levels of ~40-80 mg/dl (or under 100 mg/dl) and diabetic rats reached blood glucose levels of ~200 mg/dl or less (preferably under 200 mg/dl). Once appropriate blood glucose levels were obtained, rats were re-anesthetized with 2% isoflurane in oxygen and a 25-gauge butterfly needle was reinserted into the tail vein as
previously described. The syringe containing saline was replaced with a syringe containing 50% dextrose in saline. This solution was made prior by sterilizing sodium chloride via autoclaving and adding it to sterile 50% dextrose in water. Typically, 0.36mg of NaCl was added to 40 ml of 50% dextrose solution resulting in 50% dextrose in a 0.9% NaCl solution (normal saline). The dextrose solution was infused slowly into the tail vein to allow proper equilibration of analytes and fluid volume. Both non-diabetic and diabetic rats received dextrose dosages of 2.0 g/kg. After infusion, the animal was again removed from anesthesia and allowed to wake up. Blood glucose measurements were then taken every 10 minutes for at least 30 minutes.

13.2.8: Transmitter and rat jacket exchanges

All Medtronic Minimed® transmitters have a battery life of approximately 2 weeks. GST transmitters, which only transmit data, must therefore be exchanged and charged within this timeframe. While iPro transmitters have a battery life of ~2 weeks, they can only store one week’s worth of data. Therefore, sensors for the one-month time point will have at least one transmitter and jacket exchange before the completion of the study. Rats were brought to the OR and anesthetized with 2% isoflurane in oxygen. The current Vetrap 3M rat jacket was then carefully removed with surgical scissors. Extreme care was taken to not disturb the underlying sensors or transmitters. The sensors were examined for stability and/or extrusion. Additional sutures were used to secure the plastic housing to the skin as necessary. Transmitters were carefully removed from the
sensors and set aside. GST transmitters were carefully cleaned with alcohol wipes and then charged. iPro transmitters were carefully cleaned with alcohol wipes and set aside for later retrieval of glucose sensor data. In the case of GST transmitters, one fully-charged transmitter was attached to each glucose sensor. For iPro transmitters, one fully-charged and time-stamped iPro transmitter without initialization was attached to each glucose sensor. (iPro transmitters with initialization are only used for the first 7 days after implantation.) As before, transmitters displayed a green, blinking dot if attached to a functional, hydrated sensor. The transmitter serial number, time of initial blinking, and rat identity were recorded. A new jacket was then placed on the rat as previously described. Animals were allowed to breathe 100% oxygen until spontaneous movement was detected. The animals were then returned to their cage and monitored until sternal recumbency was maintained before being returned to the Vivarium.

13.2.9: Data acquisition from transmitters

GST transmitters transmit glucose sensor data directly to a computer via a Comlink (Medtronic®, Northridge, CA). Raw data files were generated each time the program Transmitter Utility was opened. If the program was left on continuously, as was most often the case for long-term studies, one data file was created per day. For iPro transmitters, which record data in contrast to transmitting data, raw data was manually retrieved using the iPro dock and connecting cable. Proprietary software was used to download the data, creating raw data files identical to those for the GST transmitters.
As opposed to one data file per session or day however, one raw data file was created for the duration the transmitter was connected to a functional sensor (For example, a 3-day rat would have one data file for the entire 3-day duration. One-week and one-month rats would have one or 4 data files each containing 7 days of glucose sensor data.) Using proprietary algorithms, these raw amperage values were converted into sensor glucose values, which were then used to evaluate sensor performance.

13.2.10: Explantation and tissue preparation

After 3 days, 1 week, or 1 month, animals were brought to the OR and anesthetized using 2% isoflurane in oxygen. Rat jackets were carefully removed using surgical scissors. Transmitters were removed from sensors, carefully cleaned with alcohol wipes, and set aside for charging and/or data acquisition. Fine surgical scissors were then used to gently slide under the plastic housing of Enlite™ and Enlite 2™ sensors and cut the sensor (Figure 29). The plastic housing was then carefully removed, leaving the sensor in the subcutaneous tissue. Surgical scissors were then used to cut the Sof™ sensor at the plastic housing, also leaving the sensor embedded within the tissue. Larger surgical scissors were then used to widely excise the rat pelt, taking care to ensure the sensors did not extrude during this process. Tissue surrounding the embedded sensors was trimmed into approximately 1 x 1 inch squares. The tissue squares were then laid flat in sheets of aluminum foil, wrapped carefully, and flash frozen with liquid nitrogen.
Tissue samples were stored in an -80°C freezer until further use. Animals were then sacrificed with an intracardic injection of 3M potassium chloride (KCl).

**Figure 29: Explantation procedure.** A) Surgical scissors are used to separate the sensor from the plastic housing. B) Sof™ sensor at 4 weeks visible in subcutaneous tissue when skin is reflected back. C) Enlite™ sensor at 4 weeks visible in subcutaneous tissue when skin is reflected back.

### 13.2.11: Cryosectioning

Frozen tissue samples were sectioned using the Cryotome FSE (Serial # CC0271D131) purchased from ThermoScientific®. Cryotome settings optimal for sectioning rat skin are listed as follows: chamber temperature (-25°C), cryobar temperature (-40°C), and specimen head temperature (-30°C). Six 10-µm sections per tissue sample were taken, mounted on Superfrost plus microscope slides, and stored in microscope slide boxes in an -80°C freezer until further use. These sections were taken of the implanted glucose sensors in cross-section.
13.2.12: Histology

13.2.12.1: Masson’s trichrome staining

Masson’s trichrome staining kit was purchased from Sigma-Aldrich (St. Louis, MO).

Frozen slides were first placed in a Coplin jar in Bouin’s solution overnight at 4°C. Slides were then removed from Bouin’s solution and rinsed with DI water to remove the yellow stain. The slides were allowed to dry under the hood for 30 minutes. Slides were then placed in Weigert’s Hematoxylin for 5 min and rinsed with running tap water for 5 minutes. Afterwards, slides were transferred to 100% Biebrich Scarlet-acid Fuchsin solution (RED stain) for 1 minute. The slides were then rinsed with DI water three times in separate Coplin jars. Slides were then transferred to YELLOW stain for 5 minutes, followed immediately by 10% aniline blue solution (BLUE stain) for 1 minute. YELLOW solution was prepared by mixing 50 ml phosphotungstic acid with 50 ml phosphomolybdic acid and 100 ml of deionized (DI) water. The slides were then rinsed three times in 1% acetic acid using different Coplin jars, leaving the slides in the last Coplin jar for 2 minutes. 1% acetic acid solution was prepared from 17N glacial acetic acid (GAA) by adding 11.6 ml of GAA to 188.4 ml DI water (to make 200 ml of a 1N solution). 35.2 ml of the 1N AA solution was then added to 164.8 ml of DI water to make 200 mL of 1% AA solution. After rinsing in acetic acid, the slides were then dehydrated consecutively through 75%, 90%, and 100% ethanol. Slides were rinsed with Citrisolv rinse and then placed in Citrisolv for 2 minutes. Slides were then dipped in 100% ethanol twice and allowed to dry for 1-2 minutes before mounting. Cytoseal glue
was used to secure micro cover glass slips to the microscope slides. After carefully pressing to remove air bubbles, the slides were allowed to dry before imaging. All samples were imaged with the skin surface oriented to the left and the subcutaneous space oriented to the right.

13.2.12.2: Hematoxylin & eosin staining

Hematoxylin and eosin staining kit was purchased from Sigma-Aldrich (St. Louis, MO). Frozen slides were first placed in acetone for 20 minutes. The slides were then removed and allowed to dry under a fume hood for 1 hour. Slides were transferred to Mayer’s hematoxylin for 2 minutes and then rinsed in tap water until water became clear (~5min). Slides were placed in Eosin stain for 45 seconds, and then consecutively rinsed for 2-3 seconds in 75% and 80% ethylic alcohol. Slides were then consecutively rinsed for 2-3 seconds in 90% and 95% alcohol before being immersed in 100% alcohol for 5 minutes. Slides were transferred to Citrisolv solution for 5 minutes and then consecutively rinsed in 75% and 100% alcohol. Alcohol was carefully wiped off the back of the slide, taking care not to disturb the tissue sample or to let it dry out. Cytoseal glue was applied to the specimen, and a micro cover glass slip was adhered to the microscope slide. After air bubbles were removed, the slides were allowed to dry before imaging. All samples were imaged with the skin surface oriented to the left and the subcutaneous space oriented to the right.
13.2.12.3: CD31 & Hoechst staining

Primary antibody diluting buffer (donkey serum) and secondary antibody (donkey –
mouse coupled with Dy Light-488) were purchased from Jackson Immunoresearch
(West Grove, PA). Primary antibody (mouse monoclonal) was purchased from BD
Biosciences (San Jose, CA). Hoechst 33342 was purchased from Sigma-Aldrich (St.
Louis, MO). Frozen slides were first fixed in acetone for 20 minutes in a 4°C
refrigerator. Slides were then removed from acetone and allowed to dry under a fume
hood for 1 hour. A liquid blocking pen was used to encircle the tissue samples. After 1
hour, the samples were washed 1x with 1x PBS (−/−) for 5 minutes. PBS was removed via
pipette and replaced with the primary antibody (PA) diluting buffer (5% donkey serum)
for 30 minutes. The buffer was then replaced with the primary antibody (diluted 1:200
in PA diluting buffer) for 1 hour. Tissue samples were rinsed 3x for 5 minutes each with
PBS. Secondary antibody (diluted 1:100 in PA diluting buffer) was then applied to the
tissue samples and allowed to incubate for 30 minutes in the dark. The slides were then
rinsed with PBS in the dark for 5 minutes. Hoechst solution (1 mg Hoechst 33342 in 4 ml
DI water, diluted 1:100 in dPBS) was applied to the samples for 5 minutes in the dark.
The slides were again washed for 5 minutes with PBS in the dark and then fixed for 15
minutes with 4% paraformaldehyde (PFA) on ice, in the dark. Slides were then stored in
1% PFA in the dark before imaging within 1-2 days. All samples were imaged with the
skin surface oriented to the left and the subcutaneous space oriented to the right.
13.2.12.4: CD68 macrophage staining

CD68 macrophage staining was performed on a smaller subset (n=3 for each sensor type and group) of sensors. Primary antibody (mouse anti-rat CD68, MCA341R) was purchased from AbD Serotech (Raleigh, NC). Primary antibody diluting buffer (donkey serum) and secondary antibody (donkey – mouse coupled with Dy Light-488) were purchased from Jackson Immunoresearch (West Grove, PA). Hoechst 33342 was purchased from Sigma- Aldrich (St. Louis, MO). Frozen slides were first fixed in acetone for 20 minutes in a 4°C refrigerator. Slides were then removed from acetone and allowed to dry under a fume hood for 30 minutes. A liquid blocking pen was used to encircle the tissue samples. After 30 minutes, the samples were washed 1x with 1x TBS for 5 minutes. TBS was removed via pipette and replaced with the primary antibody (PA) diluting buffer (5% donkey serum) for 45 minutes. The buffer was then replaced with the primary antibody (diluted 1:100 in PA diluting buffer) for 2 hours at room temperature. Tissue samples were rinsed 3x for 5 minutes each with TBS. Secondary antibody (diluted 1:50 in PA diluting buffer) was then applied to the tissue samples and allowed to incubate for 1 hour in the dark. Tissue samples were rinsed 3x for 5 minutes each with TBS in the dark. Hoechst solution (1 mg Hoechst 33342 in 4 ml DI water, diluted 1:100 in TBS) was applied to the samples for 5 minutes in the dark. The slides were again washed for 5 minutes with TBS in the dark and then fixed for 15 minutes with 4% paraformaldehyde (PFA) on ice, in the dark. Slides were then stored in 1% PFA
in the dark before imaging within 1-2 days. All samples were imaged with the skin surface oriented to the left and the subcutaneous space oriented to the right.

**13.2.13: Histological image analysis**

**13.2.13.1: Collagen thickness and density**

Images of Masson’s trichrome and H&E stained samples were collected using the 20x objective on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS-2Mv Digital Camera (Nikon Inc., Melville, New York). Collagen capsule thickness was measured from trichrome-stained tissue sample (Figure 30). The foreign body collagen capsule was defined as the region of dense collagen oriented parallel to the implant. In some cases, this dense collagen began at the edge of the implant. In other cases, the implant was surrounded by great numbers of inflammatory cells and the appearance of collagen was observed past the area of inflammation. The end of the capsule thickness was determined to be where collagen was no longer the primary tissue constituent (indicated by a decrease in the density of blue-green stain) nor oriented parallel to the implant surface. A minimum of seven capsule thickness measurements were taken radially around the implant cross-section and averaged for each tissue sample. The density of the collagen in the capsule was calculated in four 50 x 50 μm² fields (50 x 100 μm² if possible) per tissue sample using a previously developed MATLAB (The MathWorks, Natick, MA) program that determines the percent collagen. The program defines collagen from trichrome-stained images by the characteristic blue-green color, divides
the number of collagen positive pixels by the total pixels in the image, and multiplies this ratio by 100 to obtain percent collagen.

Figure 30: Collagen analysis. A) Masson’s trichrome-stained tissue sample with collagen capsule length measurements made at several points around the sensor. B) Cropped image of collagen capsule used for the calculation of the collagen density index (CDI).

13.2.13.2: Microvessel density

Images of the CD31 stained samples were collected the 20x objective on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS-2Mv Digital Camera (Nikon Inc., Melville, New York). FITC filter was used to obtain the fluorescent images. Once images were obtained, the microvessels were quantified in 3 “ring-like” zones beginning from the edge of the implant and moving outward. These zones were 0-50 μm, 50-100 μm, and
100-200 \( \mu m \) from the edge of the implant. Images were first thresholded and binarized using the program ImageJ (National Institutes of Health, Bethesda, MD) (Figure 31). The image is then filtered to discard remaining background and to create compact structures. An overall mask representing the entire outline of the implant is then created by tracing the outer edges of the implant. “Zoned masks” or distance maps are then created that allow for the selection of microvessels at a set distance from the edge of the original mask. Negative distance maps allow quantification of the vasculature outside of the implant. Using the grayscale/micrometer distance calibration, an area outside the implant is chosen. The thresholding tool is then used to select the respective distance range (e.g. 0-50 \( \mu m \) from the implant edge). The zoned masks are then overlaid on the binarized vessel image, and the stained structures within the selected areas are counted by selecting ‘Analyze particles.’ The number of microvessels (count number) and the total area of interest can be used to calculate the number of microvessels per pixel. Using the appropriate conversion factor (0.469 \( \mu m / \text{pixel} \) for 20x objective), microvessel density can then be expressed in microvessels/mm\(^2\).
Figure 31: Image Analysis for CD31-stained images. A) Original image showing microvessels. B) Thresholded image generated using ImageJ. C) Created mask representing the area of the sensor. D) Distance map of 0-50 µm from the sensor. E) Distance map of 50-100 µm from the sensor.

To serve as a reference, the number of microvessels in native tissue was also calculated.

Native tissue at least 250 µm away from the sensor and unaffected by the foreign body response was chosen and analyzed for microvessel density.
13.2.13.3: Inflammatory cell density

Images of Hoechst 33342 stained samples were collected using the 20x objective on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS-2Mv Digital Camera (Nikon Inc., Melville, New York). DAPI filter was used to obtain the fluorescent images. Once images were obtained, inflammatory cells were quantified in 3 “ring-like” zones beginning from the edge of the implant and moving outward. These zones were 0-50 μm, 50-100 μm, and 100-200 μm from the edge of the implant. Images were first thresholded and binarized using the program ImageJ (National Institutes of Health, Bethesda, MD). The image is then filtered to discard remaining background and to create compact structures. An overall mask representing the entire outline of the implant is then created by tracing the outer edges of the implant. “Zoned masks” or distance maps are then created that allow for the selection of cells at a set distance from the edge of the original mask. Negative distance maps allow quantification of cell density outside of the implant. Using the grayscale/micrometer distance calibration, an area outside the implant is chosen. The thresholding tool is then used to select the respective distance range (e.g. 0-50 μm from the implant edge). The zoned masks are then overlaid on the binarized cell image, and the stained structures within the selected areas are counted by selecting ‘Analyze particles.’ The number of inflammatory cells (count number) and the total area of interest can be used to calculate the number of cells per pixel. Using the appropriate conversion factor (0.469μm/pixel for 20x objective),
inflammatory cell density can then be expressed in cells/mm². This procedure is identical to the analysis described for CD31-stained images (Figure 31). To serve as a reference, the number of total cells in native tissue was also calculated. Native tissue at least 250 µm away from the sensor and unaffected by the foreign body response was chosen and analyzed for cell density.

13.2.13.4: Macrophage density

Images of CD68 stained samples were collected using the 20x objective on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS-2Mv Digital Camera (Nikon Inc., Melville, New York). FITC and DAPI filters were used to obtain the fluorescent images. Once images were obtained, macrophages were quantified in 3 “ring-like” zones beginning from the edge of the implant and moving outward. These zones were 0-50 µm, 50-100 µm, and 100-200 µm from the edge of the implant. Images were first thresholded and binarized using the program ImageJ (National Institutes of Health, Bethesda, MD). The image is then filtered to discard remaining background and to create compact structures. An overall mask representing the entire outline of the implant is then created by tracing the outer edges of the implant. “Zoned masks” or distance maps are then created that allow for the selection of cells at a set distance from the edge of the original mask. Negative distance maps allow quantification of the macrophages outside of the implant. Using the grayscale/micrometer distance calibration, an area outside the implant is chosen. The thresholding tool is then used to
select the respective distance range (e.g. 0-50 μm from the implant edge). The zoned masks are then overlaid on the binarized cell image, and the stained structures within the selected areas are counted by selecting ‘Analyze particles.’ The number of macrophages (count number) and the total area of interest can be used to calculate the number of cells per pixel. Using the appropriate conversion factor (0.469μm/pixel for 20x objective), macrophage density can then be expressed in cells/mm². This procedure is identical to the analysis described for CD31-stained images (Figure 31). To serve as a reference, the number of macrophages in native tissue was also calculated. Native tissue at least 250 μm away from the sensor and unaffected by the foreign body response was chosen and analyzed for cell density.

13.2.14: Statistical analysis

Because many statistical tests assume that data are sample from a Gaussian (normal) distribution, data were tested for normality prior to analysis. Data were tested informally for normality via creation of histograms. Generated histograms were compared to the typical Gaussian (bell-shaped curve). The Shapiro-Wilk test was also utilized to determine normality of the data. The null hypothesis of the Shapiro-Wilk test is that the population is normally distributed. If the p-value is less than the chosen alpha value, the null hypothesis is rejected and the data tested are not from a normally distributed population. P-values obtained from the data were less than the alpha value of 0.05, therefore it was concluded that the data were not normally distributed.
Additionally, quantile-quantile (QQ) plots were created using GraphPad Prism 6 (LaJolla, CA) to visually assess normality of all data. An example of a QQ plot can be viewed in Figure 32. The data plotted represent microvessel counts 0-50 μm from non-diabetic Enlite sensors (n=12). The Y axis plots the actual values while the X axis plots predicted values. The predicted values are calculated by first assuming the distribution is Gaussian (with the mean and standard deviation of the data). N (the number in the data set) points are then selected from the Gaussian distribution with equally spaced percentiles. Each predicted value becomes a Y value matched by rank with one of the actual values which is the corresponding X value. If the data are sampled from a Gaussian distribution, the QQ plot will be linear. If the X and Y values are comparable, the points are expected to line up on the line of identity (dotted line). Systematic deviation from this ideal is evidence that the data are not normal as shown in Figure 32. Because the data were not normal, they were analyzed using the Mann-Whitney U Test (also known as the Wilcoxon rank-sum test), which is a nonparametric test for unpaired data.
Figure 32: Quantile-quantile (QQ) plot for visually assessing normality of data. The data points above represent microvessel counts for non-diabetic Enlite sensors at 3 days. Because the data are not linear, the distribution is not Gaussian (normal).

13.2.15: Sensor performance analysis

Data files generated by the Carelink website and Smurf Utility program (Medtronic®, Northridge, CA) were sent to Medtronic, Inc. for data processing and analysis. Using blood calibration data, raw amperage values from the sensors were filtered and converted into sensor glucose (SG) values using proprietary algorithms specific to each sensor (Sof, Enlite, Enlite 2). An example of the resulting processed data file can be seen
in Appendix C. In addition to the process data files, sensor performance graphs were generated showing raw sensor signal and computed sensor glucose values for the lifetime of each sensor, as well as during insulin/glucose bolus events. Sensor performance data was not available for Enlite sensors implanted in diabetic animals due to high blood glucose levels. Medtronic glucose sensors were designed specifically for the blood glucose range of 40–400 mg/dL. The Sof and Enlite 2 sensors have algorithms capable of calibrating and processing data at blood glucose levels greater than 400 mg/dL, however the Enlite sensor does not. Therefore this sensor group was excluded from the sensor performance analysis.

Sensitivity to glucose (nA/mg/dL) was computed by dividing the filtered sensor response (in nA) by the blood glucose calibration value at the same timepoint. Sensitivity comparisons were made between sensors over the lifetime of the sensors using daily blood calibration values and blood measurements taken during bolus events.

Bolus events were used for evaluation of lag time and mean absolute relative difference (MARD%). Some bolus event graphs contain edited sensor response values. The algorithms used to compute sensor glucose (SG) have logic to track for abrupt changes in raw sensor response current and filter out these values. Conditions that can trigger
this filtering can be viewed in the proprietary Appendix D. Lag time was computed for complete bolus events. For each filtered sensor response (Response_in_nA) paired with a measured blood glucose value (BG), five filtered responses are calculated which are the five response current values obtained for the next 25 minutes after the initial response. Computed value of glucose can be calculated for each filtered response using the following equation:

\[
\text{Computed Glucose} = (\text{Response}_{\text{in nA}}) \times (\text{Previous_slope})
\]

The five computed glucose values are then compared to the blood glucose (BG) value paired with the initial response. The lag time can be calculated by comparing and selecting the \( \text{Comp}_\text{SG}_n \) closest to BG.

If \( \text{Comp}_\text{SG}_1 \) is closest, the lag time is \((T+5\text{mins})\). If \( \text{Comp}_\text{SG}_2 \) is closest, the lag time is \((T+10\text{mins})\). If \( \text{Comp}_\text{SG}_3 \) is closest, the lag time is \((T+15\text{mins})\). If \( \text{Comp}_\text{SG}_4 \) is closest, the lag time is \((T+20\text{mins})\). If \( \text{Comp}_\text{SG}_5 \) is closest, the lag time is \((T+25\text{mins})\).

Average lag time for each complete bolus was calculated for each sensor. The mean absolute relative difference (MARD%) was also determined for each complete bolus using the following equation:

\[
\text{MARD} = 100 \times \frac{|\text{SG} - \text{BG}|}{\text{BG}}
\]

**13.2.16: Rat Hemoglobin A1c Assay**

To further validate the diabetic rat model developed in Aim 1 and utilized in Aims 2 and 4, a rat hemoglobin A1c kit (Crystal Chem, Inc., Downers Grove, IL) was used for the
quantitative determination of hemoglobin A1c (HbA1c) in rat whole blood. A total of 8 male CD rats were used for the HbA1c assay. Whole blood from the tail vein was collected from each rat on day 0. Four of the eight rats were then given STZ injections until diabetes was achieved. Whole blood was collected from all rats at 3 weeks and 7 weeks for assessment of HbA1c.

The rat hemoglobin A1c kit is an enzymatic assay in which lysed whole blood samples are subjected to extensive protease digestion. This process results in the release of amino acids, including glycated valines from the hemoglobin beta chains. Glycated valines serve as substrates for specific frutosyl valine oxidase (FVO) enzyme. FVO cleaves N-terminal valines and produces hydrogen peroxide, which is measured using a horseradish peroxidase (POD) catalyzed reaction and a suitable chromogen. Rat hemoglobin A1c kit was purchased from Crystal Chem, Inc (Downers Grove, IL). The kit includes lysis buffer, three liquid reagents, and two lyophilized calibrators stored at 2-8°C. All reagents were provided ready-to-use and should be brought to room temperature for at least 30 minutes prior to use. Calibrators were reconstituted with 0.5 mL distilled water. Lysate was prepared by dispensing 125 µl of lysis buffer into a clean glass tube. Ten µl of fully resuspended whole blood sample, calibrator, or control was then added to the lysis buffer. The hemolysate was mixed gently by pipetting or vortex and allowed to incubate at room temperature for at least 10 minutes or until red blood
cells were completely lysed. The same procedure was repeated for all samples, calibrators, and controls. Using a 96-well microplate, 112 µl of reagent CC1a and 48 µl of reagent CC1b were added to each well (as needed) and mixed well. Twenty-five µl lysate of sample, calibrator, or control was added to each well. All samples were run in triplicate. The microplate was then placed in an incubator (37°C) for 5 minutes and allowed to equilibrate. A₇₀₀ absorbance values were then obtained using a plate reader. Seventy µl of reagent CC2 was then added to each well, and the plate was returned to the incubator for 3 minutes. A₇₀₀ absorbance values were again recorded. To determine the HbA1c concentration, the change in absorbance ΔA (0sec ~ 180sec) was calculated using the following equation:

$$\Delta A = (OD_{700\text{nm, 180sec}}) - (OD_{700\text{nm, 0sec}})$$

The HbA1c calibration curve was constructed by plotting the mean change in absorbance value for each calibrator on the Y axis versus the corresponding HbA1c concentration (5.5% or 9.5%) on the X axis. Rat HbA1c concentrations in the samples were interpolated using the calibration curve and mean absorbance values for each sample. The rat hemoglobin A1c assay has a linear range from 3.5% - 13% and has a within-run and total precision of CV < 10%.

### 13.2.17: Study Design

A sample size of 10 percutaneously-implanted, functional glucose sensors was chosen for each timepoint and each tissue type. For example, histological analysis was
performed on 10 functional Enlite sensors in diabetic tissue for the 3-day timepoint.

Originally, twenty rats were designated for each timepoint (half non-diabetic, half diabetic), however due to some instances of sensor extrusion and some animal deaths, more rats were necessary. Therefore, for percutaneously-implanted functional glucose sensors, the sample size used for histological analysis was at least n=10 for each tissue type and timepoint. Nonfunctional, fully-implanted subcutaneous sensors were implanted as available with sample sizes ranging from n=0 to n=7 depending on tissue type and timepoint. Error bars representing standard error of the mean are present in graphs for sample sizes of at least n=3.
13.3: Results

All tissue samples were evaluated histologically for collagen capsule formation, collagen density index of any encapsulation, microvessel density adjacent to the implanted sensors, and inflammatory cell density adjacent to the implanted sensors. A smaller subset of tissue samples (n=3 for each functional sensor type and timepoint) was analyzed for macrophage density adjacent to the sensor surface. All tissues were sectioned in cross-section through the sensor. Images were taken with the skin surface oriented to the left of the image and the subcutaneous space oriented to the right as shown in Figure 33.
Figure 33: Example of Masson’s trichrome-stained tissue sample showing location of Enlite sensor in subcutaneous tissue. As with all images, skin surface is oriented to the left of the image while subcutaneous space is oriented towards the right.

Sensor performance was analyzed by measuring the sensitivity to glucose (nA/mg/dl) of each functional sensor type for the lifetime of the sensor, lag time during bolus events, and mean absolute relative deviation (MARD) during bolus events.

13.3.1: Collagen Capsule Thickness and Collagen Density Index

13.3.1.1: Histology

Tissue samples were stained using Masson’s trichrome for analysis of collagen content and collagen density. Changes in collagen content for the percutaneously-implanted, functional sensors over time can be seen in Figure 34. At the 3-day timepoint, collagen capsules were beginning to form for all sensor types. As time progressed, inflammation became a prominent factor in collagen capsule formation as evidenced by the red/brown stained cell nuclei surrounding the implant. In many cases, collagen encapsulation was evident, but capsule formation began at the edge of the layer of inflammatory cells surrounding the implant.
Enlite 2 sensors were evaluated at the 1-week timepoint only. These sensors differ from the Enlite sensors in that they contain only the electrode portion of the sensor with no tube encapsulating the sensing portion. Materials exposed to the tissue for the Enlite 2 sensor are polyimide on the reverse side and a polyurethane block co-polymer on the electrode side. Images of non-diabetic tissue samples at 1 week (Figure 35) seem to suggest that Enlite 2 sensors produce a greater inflammatory response than Enlite sensors, which may be a result of tissue contact with the bare electrode.
Figure 35: Comparison of collagen content of percutaneously-implanted Enlite and Enlite 2 sensors in non-diabetic tissue at 1 week. Scale bar is 200µm for all images.

The Enlite 2 sensor and subcutaneous sensors were also analyzed for collagen content at the 1-week timepoint in diabetic tissue (Figure 36). The fully-implanted subcutaneous Sof sensor produced a considerable inflammatory response, which was concentrated primarily at the site of the sensing electrode. The subcutaneous Enlite sensor had disorganized collagen formation and evidence of inflammatory cell infiltration.
At the 1-month timepoint, fully-implanted subcutaneous Enlite sensors were compared with their functional counterparts (Figure 37). At 1-month, all sensors types were surrounded by an aggressive inflammatory response except for the non-diabetic subcutaneous Enlite sensor. In the other sensors, little collagen development was seen in the regions directly adjacent to the sensor surface. These images seem to indicate that the foreign body response around the sensors does not resolve over time, but continues in a chronic inflammatory state.
Figure 37: Comparison of collagen content of percutaneously and fully-implanted (SubQ) sensors in non-diabetic tissue at 1 month. Scale bar is 200µm for all images.

13.3.1.2: Collagen Capsule Thickness

All tissue samples were analyzed for collagen encapsulation surrounding the sensor. Some sensors, particularly those surrounded considerably by inflammatory cells, did not develop collagen capsules. The following graphs are therefore divided into graphs showing collagen thicknesses for all sensors (with a thickness of zero designated for sensors without a capsule) and graphs showing collagen thicknesses for encapsulated
sensors only. Collagen density index (CDI) measurements were taken only from sensors with collagen encapsulation.

No significant differences in collagen capsule thickness were observed at any timepoint when comparing the non-diabetic and diabetic response to each sensor type, including sensors without capsules (Figure 38).
Figure 38: Comparison between non-diabetic and diabetic collagen thickness for all sensors at all timepoints. 3-day diabetic Sof SQ sensors and 1-week non-diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

When only encapsulated sensors were considered, no significant differences in collagen capsule thickness were again observed when comparing the non-diabetic and diabetic response at all timepoints (Figure 39).
Figure 39: Comparison between non-diabetic and diabetic collagen thickness for encapsulated sensors (sensors with a collagen capsule). 3-day diabetic Enlite SQ and Sof SQ sensors and 1-week non-diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

Sensors were also analyzed to determine if differences in collagen capsule thickness existed between sensors in the diabetic and non-diabetic groups (Figure 40). At 3 days, a significant difference (p = 0.021) was observed between percutaneously-implanted diabetic Enlite and Sof sensors when all sensors were considered. This difference was not observed at later timepoints. At 1 week, significant differences were observed in the non-diabetic group between the percutaneously-implanted Enlite 2 and Enlite sensors (p
= 0.040) and Enlite 2 and Sof sensors (p = 0.001). Both Enlite and Sof sensors had a significantly thicker collagen capsule than the Enlite 2 sensor. No significant differences were observed among sensors at the 1-month timepoint.

Figure 40: Comparison of collagen thickness between all sensors at all timepoints. Asterisk (*) indicates p < 0.05. 3-day diabetic Sof SQ sensors and 1-week non-diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

When only encapsulated sensors were considered (Figure 41), significant differences were observed among sensor groups at the 1-week timepoint. In the non-diabetic
group, encapsulated Sof (Per.) sensors had significantly thicker (p = 0.002) collagen capsules than percutaneous Enlite 2 sensors. In the diabetic group, percutaneous Enlite sensors had significantly thicker collagen capsules than percutaneous Enlite 2 (p = 0.018) sensors and fully-implanted Enlite (SQ) sensors (p = 0.010). Additionally, the diabetic Sof (Per.) sensors had significantly thicker collagen capsules than the subcutaneous Enlite sensors (p = 0.003). No significant differences were observed among encapsulated sensors at the 3-day and 1-month timepoints.
Figure 41: Comparison of collagen thickness between encapsulated sensors (sensors with a collagen capsule) at all timepoints. All symbols (▲, !, *) indicate p < 0.05. 3-day diabetic Enlite SQ and Sof SQ sensors and 1-week non-diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

Sensor capsule thickness was also analyzed over time to see if any noticeable changes occurred as the foreign body response progressed (Figure 42). When all sensors were considered, a significant difference was observed for the non-diabetic Sof (Per.) sensor between the 3-day and 1-week timepoints (p = 0.036). This difference was not observed in only encapsulated sensors however. When excluding sensors without capsules, the diabetic Enlite (Per.) sensors had a significant increase in collagen capsule thickness.
from 3 days to 1 week (p = 0.030). In general, collagen capsule thickness for all sensors tended to increase over time, though these changes were not significant.

Figure 42: Collagen thickness over time for all sensors (left) and encapsulated sensors (sensors with collagen capsules) (right) Asterisk (*) indicates p < 0.05. 3-day diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

13.3.1.3: Collagen Density Index (CDI)

Collagen density index (CDI) measurements were taken from Masson’s trichrome-stained tissue samples of encapsulated sensors only. No significant differences were observed at any timepoint when comparing CDI of all sensor types in non-diabetic and diabetic tissues (Figure 43).
Figure 43: Comparison between non-diabetic and diabetic collagen density index (CDI) for encapsulated sensors (sensors with collagen capsules). 3-day diabetic Enlite SQ and Sof SQ sensors and 1-week non-diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

When comparing sensor types to each other, significant differences in collagen density were observed at the 1-week timepoint (Figure 44). In the non-diabetic group, the Sof (Per.) sensors had significantly greater collagen density than the Enlite 2 (Per.) sensors (p = 0.001). In the diabetic group, the percutaneous Enlite sensor had significantly greater collagen density than the percutaneous Enlite 2 sensor (p = 0.018). No significant
differences in collagen density were observed between sensors at the 3-day or 1-month timepoints (Figure 44).

Figure 44: Comparison of collagen density index (CDI) between encapsulated sensors (sensors with a collagen capsule) at all timepoints. Asterisk (*) indicates $p < 0.05$. 3-day diabetic Enlite SQ and 1-week non-diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

Collagen density index was also analyzed over time in order to see if significant changes occurred (Figure 45). In general, CDI for each sensor type remained fairly constant over time with no significant differences observed between timepoints. The non-diabetic percutaneous Sof sensor however, had a significant decrease in collagen density over...
time. No significant difference was observed between the 3-day and 7-day timepoint for the non-diabetic Sof Per. sensor, however both of these timepoints had a significantly higher CDI than the 1-month timepoint (p = 0.004, p = 0.010).

Figure 45: Collagen density index (CDI) of encapsulated sensors (sensors with collagen capsules) over time. Asterisk (*) indicates p < 0.05. 3-day diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

13.3.2: Inflammatory Cell Density

13.3.2.1: Histology

Inflammatory cell density can be viewed in tissue samples via staining with hematoxylin and eosin and via Hoechst 33342 stain. Both methods stain all cell nuclei; H&E stains cell nuclei dark purple, while Hoechst is a fluorescent cell nuclei marker. Changes in cell number seen in tissue samples can be attributed to migration of inflammatory cells.
to the sensor site as native stromal cells do not proliferate at a significant rate. Both stains are shown consecutively for comparison. Figures 46 and 47 show the change in inflammatory cell density over time for all functional, percutaneously-implanted sensor types. For all sensor types, cell density appears to increase over time, suggesting a chronic inflammatory response.

Figure 46: Inflammatory cell density over time for functional sensors as seen from hematoxylin and eosin-stained tissue samples. Scale bar is 200μm for all images.
Figure 47: Inflammatory cell density over time for functional sensors as seen from Hoechst 33342-stained tissue samples. Scale bar is 200μm for all images.

For the 1-week timepoint, percutaneous Enlite 2 sensors were compared with percutaneous Enlite sensors in non-diabetic tissue (Figure 48). Both stains show greater inflammatory cell density adjacent to the Enlite 2 sensor, again suggesting that the bare electrode promotes a more adverse tissue response.
Figure 48: Inflammatory cell density at 1 week for non-diabetic Enlite Per. and Enlite 2 Per. sensors as seen from hematoxylin and eosin-stained and Hoechst 33342-stained tissue samples. Scale bar is 200µm for all images.

At the 1-week timepoint, the percutaneous Enlite 2 sensor and subcutaneous sensors showed varying degrees of inflammation in diabetic tissue (Figures 49 and 50). The diabetic Enlite 2 sensor showed a lesser inflammatory cell density when compared to the non-diabetic Enlite 2 sensor. This result was expected since diabetics generally have more difficulty establishing an immune response. The diabetic subcutaneous Sof sensor
showed a greater inflammatory response than the percutaneous Sof sensor, though this inflammation was concentrated almost solely at the sensing electrode. It is possible that the tissue interaction with the bare electrode combined with excess hydrogen peroxide from the non-functional sensor contributed to this greater immune response.

Figure 49: Inflammatory cell density at 1 week for percutaneous Enlite 2 and subcutaneous sensors in diabetic tissue as seen from hematoxylin and eosin-stained tissue samples. Scale bar is 200µm for all images.
Figure 50: Inflammatory cell density at 1 week for percutaneous Enlite 2 and subcutaneous sensors in diabetic tissue as seen from Hoechst 33342-stained tissue samples. Scale bar is 200µm for all images.

At the 1-month timepoint, subcutaneous Enlite sensors were compared with their percutaneously-implanted, functional counterparts in both diabetic and non-diabetic tissues (Figures 51 and 52). Subcutaneous sensors similarly showed considerable inflammation, though the inflammatory response appears to be more concentrated with the subcutaneous sensors.
Figure 51: Inflammatory cell density at 1 month for percutaneous and subcutaneous Enlite sensors in diabetic and non-diabetic tissues as seen from hematoxylin and eosin-stained tissue samples. Scale bar is 200μm for all images.
13.3.2.2: Inflammatory Cell Density

Inflammatory cell density was quantified using a previously-described protocol that analyzes Hoechst 33342-stained tissue samples. Inflammatory cell counts were calculated for three zones beginning from the surface of the sensors. Figure 53 compares the inflammatory cell counts in diabetic and non-diabetic tissues for sensors at 3 days. In general, inflammatory cell counts were higher around sensors in non-diabetic tissues, though these findings are not significant. Interestingly, inflammatory cell counts in the
100-200 μm region of the percutaneous Enlite sensors were significantly greater in diabetic tissues (p = 0.005) even though this difference is not seen in more proximal zones.

Figure 53: Comparison between non-diabetic and diabetic inflammatory cell density at 3 days. Asterisk (*) indicates p < 0.05. 3-day subcutaneous Enlite sensors in the 0-50 μm and 50-100 μm zones were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

At the 1-week timepoint, no significant differences in inflammatory cell density were observed when sensors were compared in non-diabetic and diabetic tissues (Figure 54). Inflammatory cell density was relatively equal for all sensor types, but was not consistently greater in one tissue type.
Figure 54: Comparison between non-diabetic and diabetic inflammatory cell density at 1 week. Values are mean ± SEM.

Similarly to the 1-week timepoint, no significant differences in inflammatory cell density were observed at the 1-month timepoint (Figure 55).
Figure 55: Comparison between non-diabetic and diabetic inflammatory cell density at 1 month. Values are mean ± SEM.

When sensors were compared to each other with regards to inflammatory cell density, significant differences were observed at all timepoints. At the 3-day timepoint, the percutaneously-implanted Enlite sensor had a significantly greater inflammatory cell density than the percutaneously-implanted Sof sensor in the 0-50 μm zone in non-diabetic tissue (p = 0.019) (Figure 56). Similarly, the percutaneously-implanted Enlite sensor had a significantly greater inflammatory cell density than the percutaneously-implanted Sof sensor in the 100-200 μm zone in diabetic tissue at 3 days (p = 0.0003). In all zones and for both percutaneous Enlite and Sof sensors, the Enlite sensors had a greater inflammatory cell density in diabetic and non-diabetic tissue. This result is
interesting as the smaller, more flexible design of the Enlite sensor was intended to promote a lesser inflammatory response.

**3 Day Inflammatory Cell Counts - Sensor Comparison**

![Graph showing inflammatory cell counts for different sensor types and sizes](image)

Figure 56: Inflammatory cell density for all sensors at 3 days. Asterisk (*) indicates p < 0.05. Diabetic Enlite SQ sensors in the 0-50 μm and 50-100 μm zones were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

At the 1-week timepoint, differences in inflammatory cell density between sensor types appeared to even out as the acute inflammatory stage progressed (Figure 57). In the 50-100 μm zone, the diabetic Sof SQ sensors had a significantly greater inflammatory cell density than the diabetic Enlite Per. sensors (p = 0.049). This significance could be the result of a number of factors however, from difference in size, difference in stiffness,
difference in implantation method (percutaneous vs. fully-implanted), or a combination of the three.

Figure 57: Inflammatory cell density for all sensors at 1 week. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.

At the 1-month timepoint, significant differences in inflammatory cell density were observed only in the 100-200 μm zone (Figure 58). In non-diabetic tissue, the percutaneous Enlite sensors had significantly greater inflammatory cell density than the percutaneous Sof sensors (p = 0.041). In diabetic tissue, the percutaneous Enlite sensors had a significantly greater inflammatory cell density than the subcutaneous Enlite
sensors (p = 0.034), which may be a result of external mechanical stress from the attached sensor hub and transmitter.

### 1 Month Inflammatory Cell Counts - Sensor Comparison

![Bar chart showing inflammatory cell density for all sensors at 1 month. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.](image)

**Figure 58: Inflammatory cell density for all sensors at 1 month. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.**

Inflammatory cell density was also analyzed over time for all zones to determine how the inflammatory response progressed for each sensor type. Figure 59 shows the change in inflammatory cell density in the 0-50 μm zone for sensor types in diabetic and non-diabetic tissues. In non-diabetic tissue, the percutaneous Sof sensor had a significant increase in inflammatory cell density from the 3-day to the 1-month timepoint (p = 0.007). In diabetic tissue, the percutaneous Sof sensor had a significant increase in
inflammatory cell density from the 3-day to the 1-week timepoint (p = 0.029), a difference that increased at the 1-month timepoint (p = 0.003). Interesting to note however, the inflammatory cell density for the percutaneous Sof sensor in diabetic tissue did not significantly differ from native diabetic tissue, a possible indication that the diabetic inflammatory response is delayed or diminished.

Figure 59: Inflammatory cell density over time in zone 0-50μm from sensor. Asterisk (*) indicates p < 0.05. 3-day diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

In the 50-100 μm zone, inflammatory cell density increased significantly for all percutaneously-implanted functional sensors except for the non-diabetic Enlite sensor.
(Figure 60). For the non-diabetic Sof Per. sensor, inflammatory cell counts remained stable from the 3-day to 1-week timepoints, but then increased significantly at the 1-month timepoint (p = 0.048, p = 0.019). In diabetic tissue, the percutaneous Sof sensor had a significant increase in inflammatory cell density from the 3-day to the 1-week timepoint (p = 0.001), a difference that increased at the 1-month timepoint (p = 0.001). Finally, for the diabetic percutaneous Enlite sensor, inflammatory cell counts remained stable from the 3-day to 1-week timepoints, but then increased significantly at the 1-month timepoint (p = 0.017, p = 0.023). Inflammatory cell density remained relatively unchanged over time in the 50-100 μm zone for the diabetic subcutaneous Enlite sensor.
Figure 60: Inflammatory cell density over time in zone 50-100μm from sensor. Asterisk (*) indicates p < 0.05. 3-day diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

In the 100-200 μm zone, inflammatory cell counts tended to increase or remain relatively unchanged over time (Figure 61). In non-diabetic tissue, the percutaneous Enlite sensors had a significant increase in inflammatory cell density from the 3-day to the 1-month timepoint (p = 0.046). In diabetic tissue, the percutaneous Sof sensor also had a significant increase in inflammatory cell density from the 3-day to the 1-month timepoint (p = 0.007). Also in diabetic tissue, the percutaneous Enlite sensor had a
significant decrease in inflammatory cell density from the 3-day to the 1-week timepoint (p = 0.001), however cell counts increased once again by the 1-month timepoint.

Figure 61: Inflammatory cell density over time in zone 100-200μm from sensor. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.

13.3.2.3: Macrophage Immunohistochemistry

CD68 macrophage staining was also performed on a smaller subset of tissue samples in order to address the non-specificity of H&E and Hoechst 33342-staining. CD68 staining was used to specifically quantify the number of macrophages involved in the immune response at each timepoint. Using different fluorescent filters, images containing only macrophages (FITC filter) and images containing total cells (DAPI) filter can be
compared to determine the contribution of macrophages to the total immune response. The images of total cells were obtained from Hoechst 33342-stained tissue samples as described previously. CD68-stained images and corresponding Hoechst 33342-stained images in diabetic and non-diabetic tissues were taken of percutaneously-implanted, functional sensors only (Enlite Per., Sof Per., Enlite 2 Per.), with a sample size of 3 for each timepoint. Figure 62 shows the change in macrophage density over time for the percutaneous Sof and Enlite sensors. Macrophage density appears to increase over time for all sensor types and is concentrated primarily at the sensor surface.
Figure 62: Macrophage density over time for functional sensors as seen from CD68-stained tissue samples. Scale bar is 200µm for all images.

The concentration of macrophages in regards to the total amount of inflammatory cells can be determined by comparing Figures 62 and 63. Figure 62 illustrates how many of the total inflammatory cells in Figure 63 stain positive for macrophage marker.
Figure 63: Inflammatory cell density over time for functional sensors as seen from Hoechst 33342-stained tissue samples. Scale bar is 200μm for all images.

At the 1-week timepoint, the macrophage density of percutaneous Enlite 2 sensors was compared with percutaneous Enlite counterparts in both non-diabetic and diabetic tissue (Figure 64). Macrophage density appears to be greater adjacent to Enlite 2 sensors however sample size may be too small to determine significance. Macrophage concentration adjacent to Enlite 2 sensors can be compared to total inflammatory cell density in Figure 65.
Figure 64: Macrophage density for percutaneous Enlite and Enlite 2 sensors as seen from CD68-stained tissue samples. Scale bar is 200µm for all images.

Figure 65: Inflammatory cell density for percutaneous Enlite and Enlite 2 sensors as seen from Hoechst 33342-stained tissue samples. Scale bar is 200µm for all images.

13.3.2.4: Macrophage Density

Macrophages were quantified using the previously-described method for quantifying inflammatory cell counts and microvessels. Figures 66-69 show macrophage density in comparison to total cell density for all sensor types and timepoints. As expected, for all sensors and timepoints, the concentration of macrophages was greatest directly adjacent to the sensor surface and gradually decreased in zones further away from the sensor. At the 3-day timepoint, the percutaneous Enlite sensors had the greatest concentration of
macrophages directly adjacent to the sensor (0-50 μm), though these results are not significant (Figure 66).

![Figure 66: Macrophages vs. total inflammatory cells for all functional sensors at 3 days. Values are mean ± SEM.](image)

At the 1-week timepoint, macrophage density was relatively consistent among all sensor types (Figure 67). Because of the large number of sensor types, Figure 68 illustrates the macrophage density versus total cellularity for percutaneous Sof and Enlite sensors only at the 1-week timepoint.
Figure 67: Macrophages vs. total inflammatory cells for all functional sensors at 1 week. Macrophages in diabetic native tissue were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.
Figure 68: Macrophages vs. total inflammatory cells for functional Sof and Enlite sensors at 1 week. Macrophages in diabetic native tissue were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

At the 1-month timepoint (Figure 69), non-diabetic sensors had the greatest macrophage density in the 0-50 μm zone. The non-diabetic percutaneous Enlite sensor additionally had the greatest macrophage density of all sensors at all distance zones. Macrophage densities were relatively equal among diabetic percutaneous sensors at the 1-month timepoint.
Figure 69: Macrophages vs. total inflammatory cells for all functional sensors at 1 month. Values are mean ± SEM.

Alternatively, Figures 66-69 can be viewed as ratios of macrophages to total cellularity (Figures 70-72). Figure 70 depicts the ratio of macrophages to total cells for all percutaneous sensor types at 3 days. In the 0-50 μm zone, macrophage concentration was fairly consistent among sensor types (~ 0.50-0.75). The ratio of macrophages to total cells was greatest for the non-diabetic percutaneous Enlite sensor at all distance zones, however there was considerable variability in the data. As expected, all ratios decreased with increasing distance from the sensor.
Figure 70: Ratio of macrophages to total cells at 3 days for all percutaneous, functional sensor types. Values are mean ± SEM.

At the 1-week timepoint, the concentration of macrophages increased adjacent to sensors in non-diabetic tissues while decreasing slightly in diabetic tissues (Figure 71). This occurrence is consistent with macrophage activation and recruitment in a healthy immune response. In contrast, the diabetic immune response was not quite as aggressive for all sensor types, though this result was not significant.
Figure 71: Ratio of macrophages to total cells at 1 week for all percutaneous, functional sensor types. Values are mean ± SEM.

At the 1-month timepoint, macrophage concentration appeared to have stabilized, with ratios similar to those seen at the 1-week timepoint (Figure 72). For all sensor types however, approximately half of all inflammatory cells were composed of macrophages, an indication that the inflammatory response is actively persistent.
Figure 72: Ratio of macrophages to total cells at 1 month for all percutaneous, functional sensor types. Values are mean ± SEM.

Figures 73-75 illustrate the comparison of macrophage density between percutaneous sensor types at each timepoint. At the 3-day timepoint, macrophage density was greatest adjacent to percutaneous Enlite sensors in both non-diabetic and diabetic tissues (Figure 73). This result was again unanticipated since the design of the Enlite sensor was intended to promote a less aggressive foreign body response. At the 1-week timepoint, macrophage density for the percutaneous Sof and Enlite sensors remained relatively unchanged from 3-day levels (Figure 74). In diabetic tissue, macrophage density for the percutaneous Sof and Enlite sensors was slightly less than that in non-diabetic tissue, however this result was not significant. Macrophage density for the percutaneous Enlite 2 sensors was similar in non-diabetic and diabetic tissues.
Figure 73: Sensor comparison of macrophage density for percutaneous sensors at 3 days. Values are mean ± SEM.

Figure 74: Sensor comparison of macrophage density for percutaneous sensors at 1 week. Values are mean ± SEM.
Finally, at the 1-month timepoint, macrophage density was again relatively stable, with diabetic concentrations somewhat less than those in non-diabetic tissues (Figure 75).

![1 Month Macrophages - Sensor Comparison](image)

**Figure 75:** Sensor comparison of macrophage density for percutaneous sensors at 1 month. Values are mean ± SEM.

Sensor types were compared in non-diabetic and diabetic tissues to determine if differences in macrophage density existed at each timepoint. At the 3-day timepoint, the non-diabetic percutaneous Enlite sensor had a greater macrophage density in all zones in comparison to diabetic tissue, however these results were not significant possibly due to low sample size (Figure 76). Interestingly, at the 3-day timepoint, macrophage density in diabetic tissue for the percutaneous Sof sensor was equal to or greater than that of non-diabetic tissue in all zones. This result was unexpected since diabetics generally have a delayed or weaker immune response, however it is important to note that the sample size for determining macrophage density was very small.
Figure 76: Comparison between non-diabetic and diabetic macrophage density at 3 days. Values are mean ± SEM.

At the 1-week timepoint, no significant differences in macrophage density were observed between sensors in non-diabetic and diabetic tissues. Macrophage density for percutaneous Sof and Enlite sensors was greater in non-diabetic tissue than diabetic tissue. For the percutaneous Enlite 2 sensor, macrophage density was essentially equal in both tissues, with a slightly greater density in diabetic tissue (Figure 77).
Figure 77: Comparison between non-diabetic and diabetic macrophage density at 1 week. Values are mean ± SEM.

At the 1-month timepoint, macrophage density in non-diabetic tissue was equal to or greater than in diabetic tissue for both functional sensor types (Figure 78). A considerable number of macrophages were still present adjacent to sensors in diabetic tissue however, suggesting that the foreign body response, instead of resolving, is stabilizing into a chronic state.
Figure 78: Comparison between non-diabetic and diabetic macrophage density at 1 month. Values are mean ± SEM.

Changes in macrophage density were also analyzed over time for each functional sensor type. Figure 79 illustrates the change in macrophage density in the 0-50 µm zone directly adjacent to the sensor surface. In non-diabetic tissue, macrophage density increased from the 3-day to 1-week timepoints for the percutaneous Sof sensor, which is consistent with macrophage activation and recruitment in a healthy immune response. However, at the 1-month timepoint for the non-diabetic Sof sensor, macrophage density remained relatively unchanged, indicating a prolonged or unresolved foreign body response. Macrophage density for the non-diabetic Enlite sensor and the diabetic Sof sensor remained consistently high at all timepoints. In diabetic tissue, macrophage density adjacent to the percutaneous Enlite sensor decreased considerably from the 3-day to 1-week timepoints and remained stable from the 1-week to 1-month timepoints.
Figure 79: Macrophage density over time in zone 0-50 μm from sensor. Values are mean ± SEM.

As expected, macrophage density in the 50-100 μm zone was considerably less than in the 0-50 μm zone, except for the non-diabetic percutaneous Enlite sensor (Figure 80).

For the non-diabetic percutaneous Enlite sensor, macrophage density remained relatively unchanged in the two most proximal zones, suggesting considerable macrophage migration to the sensor site. Macrophage density for all sensor types remained consistently stable over time in the 50-100 μm zone.
Figure 80: Macrophage density over time in zone 50-100 μm from sensor. Values are mean ± SEM.

Changes in macrophage density over time in the 100-200 μm zone can been seen in Figure 81. As in previous zones, the greatest macrophage density was observed adjacent to the non-diabetic percutaneous Enlite sensor. No significant differences in macrophage density over time were observed for any sensor type in the 100-200 μm zone, however, macrophage density does increase considerably from the 3-day to 1-week timepoints for the diabetic percutaneous Enlite sensor.
Figure 81: Macrophage density over time in zone 100-200 μm from sensor. Values are mean ± SEM.

13.3.3: Microvessel Density

13.3.3.1: Immunohistochemistry

Tissue samples were also analyzed for microvessel density adjacent to percutaneously-implanted and fully-implanted sensors. Figure 82 illustrates the change in microvessel density over time for percutaneously-implanted functional sensors as seen from CD31-stained tissue samples.
Figure 82: Microvessel density over time for functional sensors as seen from CD31-stained tissue samples. Scale bar is 200µm for all images.

At the 1-week timepoint, microvessel density adjacent to functional Enlite 2 sensors was compared to microvessel density adjacent to functional Enlite sensors in non-diabetic tissue (Figure 83). No significant differences in microvessel density between the two sensor types can be observed visually from CD31-stained images.
Figure 83: Microvessel density at 1 week for functional Enlite and Enlite 2 sensors in non-diabetic tissue as seen from CD31-stained tissue samples. Scale bar is 200μm for all images.

Microvessel density adjacent to functional Enlite 2 sensors was also compared to microvessel density adjacent to functional Enlite sensors in diabetic tissue at the 1-week timepoint (Figure 84). Additionally, fully-implanted, subcutaneous Sof and Enlite sensors were also analyzed at the 1-week timepoint for microvessel density. Visually, microvessel density adjacent to subcutaneous sensors appears greater in comparison to their percutaneous counterparts.
Figure 84: Microvessel density at 1 week for percutaneously-implanted functional and fully-implanted nonfunctional sensors in diabetic tissue as seen from CD31-stained tissue samples. Scale bar is 200μm for all images.

At the 1-month timepoint, fully-implanted subcutaneous Enlite sensors were analyzed for microvessel density in non-diabetic and diabetic tissue (Figure 85). In both tissue types, subcutaneous sensor types appear to have a greater microvessel density. In addition, microvessel density appears noticeably greater adjacent to percutaneous and subcutaneous sensors in non-diabetic tissue in comparison with diabetic tissue.
13.3.3.2: Microvessel Density

Figures 86-88 illustrate the comparison of microvessel density adjacent to implanted sensors in non-diabetic and diabetic tissue. At the 3-day timepoint (Figure 86), no significant differences were observed in non-diabetic and diabetic tissue for any sensor type. In non-diabetic tissue, the percutaneous Enlite sensor had a microvessel density similar to native non-diabetic tissue. For all other sensor types, microvessel density was
considerably less than that seen in native tissue, however these results were not significant.

Figure 86: Comparison between non-diabetic and diabetic microvessel density at 3 days. 3-day diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

At the 1-week timepoint, a significant difference in microvessel density for the percutaneous Enlite sensor was observed at each distance zone when comparing non-diabetic to diabetic tissue (p = 0.043, p = 0.003, p = 0.029). These results were expected due to the fact that angiogenesis is a process that is deficient in diabetics. In fact, for most sensor types at 1 week, microvessel density in non-diabetic tissue was equal to or greater in comparison with diabetic tissue. For all sensor types except for the diabetic,
percutaneous Enlite sensor, no significant differences between microvessel density adjacent to the sensor and native microvessel density were observed. In diabetic tissue, microvessel density adjacent to the percutaneous Enlite sensor was significantly lower than that of native tissue (Figure 87).

![1 Week Vasculature - Non-diabetic vs. Diabetic](image)

**Figure 87**: Comparison between non-diabetic and diabetic microvessel density at 1 week. Asterisk (*) indicates $p < 0.05$. Values are mean ± SEM.

At the 1-month timepoint, no significant differences in microvessel density adjacent to percutaneously-implanted, functional sensors were observed when comparing non-diabetic and diabetic tissue (Figure 88). In the 100-200 µm zone, the microvessel density adjacent to the non-diabetic Enlite SQ sensor was significantly greater than that of its diabetic counterpart ($p = 0.016$). As expected, microvessel density for all sensor types
was lowest in the 0-50 μm zone directly adjacent to the sensor surface. In zones more distant from the sensor surface, microvessel density remained stable or increased considerably for all sensor types.

![1 Month Vasculature - Non-diabetic vs. Diabetic](image)

**Figure 88:** Comparison between non-diabetic and diabetic microvessel density at 1 month. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.

At all timepoints, microvessel density comparisons were also made among sensor types (Figures 89-91). At the 3-day timepoint, no significant differences in microvessel density among sensor types were observed, and microvessel density for all sensor types was not significantly different that than of native tissue (Figure 89).
Figure 89: Microvessel density for all sensors at 3 days. 3-day diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

At the 1-week timepoint, several significant differences in microvessel density were observed among sensor types implanted in diabetic tissue (Figure 90). For all distance zones, microvessel density adjacent to diabetic Sof Per. sensors was significantly greater than that of diabetic Enlite Per. sensors (p = 0.002, p = 0.030, p = 0.017, denoted by *). In the 0-50 μm and 100-200 μm zones, the diabetic Enlite 2 Per. sensor had a significantly greater microvessel density in comparison with the diabetic Enlite Per. sensor (p = 0.009, p = 0.022, denoted by ^). In the 100-200 μm zone, microvessel density surrounding the Sof SQ sensor was significantly greater than that of the Enlite SQ sensor (p = 0.036,
denoted by #). Finally, in the 0-50 µm and 50-100 µm zones, microvessel density was significantly greater adjacent to Sof SQ sensors in comparison with Enlite Per. sensors (p = 0.007, p = 0.007, denoted by !), however it is possible that too many variables exist to determine the true cause of significance.

![1 Week Vasculature - Sensor Comparison](image.png)

**Figure 90:** Microvessel density for all sensors at 1 week. All symbols (*, ^, !, #) indicate p < 0.05. Values are mean ± SEM.

At the 1-month timepoint, no significant differences in microvessel density were observed among sensors at any zone (Figure 91). In diabetic tissue, microvessel density adjacent to the subcutaneous Enlite sensor was significantly lower than that of native diabetic tissue for all distance zones (p < 0.05). In the 0-50 µm zone, microvessel density
adjacent to non-diabetic Enlite Per. and Sof Per. sensors was significantly lower than that of native tissue (p < 0.05). Similarly, microvessel density adjacent to diabetic Enlite Per. sensors was significantly lower than that of native tissue in the 0-50 μm zone (p = 0.004). These differences were not observed in more distant zones as microvessel density surrounding the implanted sensors appeared to equilibrate with the native tissue.

Figure 91: Microvessel density for all sensors at 1 month. Values are mean ± SEM.

Figures 92-94 depict changes in microvessel density over time for each distance zone surrounding the implanted sensors. In the 0-50 μm zone, microvessel density adjacent to percutaneous sensors in non-diabetic tissues remained relatively stable over all timepoints, however the microvessel density adjacent to the non-diabetic Enlite sensor
decreased considerably from the 3-day to 1-week timepoint. In diabetic tissue, microvessel density adjacent to the percutaneous Sof sensor steadily increased over time, while microvessel density adjacent to the percutaneous Enlite sensor remained stable between the 3-day and 1-month timepoints. A significant increase in microvessel density was observed from the 1-week to 1-month timepoints for the diabetic, percutaneous Enlite sensor (p = 0.014). Finally, microvessel density directly adjacent to the diabetic Enlite SQ sensors steadily increased over time, however this result was not significant (Figure 92).

![Vasculature over Time - 0-50μm](image)

**Figure 92:** Microvessel density over time in zone 0-50μm from sensor. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.
In the 50-100 μm zone, microvessel density adjacent to implanted sensors generally increased over time except for the non-diabetic Enlite Per. sensors, which remained relatively stable (Figure 93). Similarly to the 0-50 μm zone, microvessel density adjacent to the diabetic Enlite Per. sensors decreased from the 3-day to 1-week timepoints and significantly increased from the 1-week to 1-month timepoints (p = 0.004).

![Vasculature over Time - 50-100μm](image)

**Figure 93**: Microvessel density over time in zone 50-100μm from sensor. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.

Similar changes in microvessel density were observed in the 100-200 μm zone, with microvessel density remaining stable or steadily increasing over time (Figure 94). In non-diabetic tissue, microvessel density increased significantly between the 3-day and 1-month timepoints for the percutaneous Sof sensor (p = 0.025). In diabetic tissue,
microvessel density adjacent to the percutaneous Enlite sensors was significantly greater at the 1-month timepoint in comparison to both 3-day (p = 0.037) and 1-week (p = 0.004) timepoints.

Figure 94: Microvessel density over time in zone 100-200µm from sensor. 3-day diabetic Enlite SQ sensors were not evaluated for statistical significance due to low sample size. Asterisk (*) indicates p < 0.05. Values are mean ± SEM.

13.3.4: Sensor Performance

Sensor performance analysis was generously assisted by Medtronic, Inc. Raw glucose sensor signal data sheets (with time and amperage values) and blood calibration measurements were provided to Medtronic, Inc. for processing. Proprietary algorithms specific to each glucose sensor type were used to calibrate the sensors and compute
sensor glucose (SG) values from raw amperage data. An example of a processed data file (Excel sheet) can be viewed in Appendix B. In addition, plots of sensor signal and sensor glucose values throughout the lifetime of the sensor were provided (Figure 95). The diabetic Enlite sensors were not evaluated for sensor performance because the proprietary algorithm specific to these sensors was unable to calibrate the sensors at blood glucose levels greater than 400 mg/dl.
Figure 95: Glucose sensor plots generated by Medtronic, Inc. depicting raw sensor signal over time, computed sensor glucose values, and blood glucose measurements.

Previous studies involving Medtronic® glucose sensors have shown a linear relationship between sensor glucose values and blood glucose values \textit{in vitro}. Using sensor glucose values computed by Medtronic, Inc. and corresponding blood glucose values, linear regression was used to determine $r^2$ values for each functional sensor type \textit{in vivo} (Table 12). Very important to note, the relationship between sensor glucose values and blood glucose values is \textit{not} linear \textit{in vivo}, as can be seen by the considerably
low $r^2$ values. Proprietary algorithms are thus necessary in order to computer sensor glucose values from blood glucose measurements.
Table 12: Correlation between sensor glucose values (SG) and blood glucose values (BG) in vivo.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic Sof</th>
<th>Diabetic Sof</th>
<th>Non-diabetic Enlite</th>
<th>Non-diabetic Enlite 2</th>
<th>Diabetic Enlite 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r^2$ (mean ± SEM)</td>
<td>0.256 ± 0.115</td>
<td>0.395 ± 0.139</td>
<td>0.536 ± 0.202</td>
<td>0.401 ± 0.134</td>
<td>0.429 ± 0.143</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Sensor life for all functional sensors is listed in Table 13. Sensor data for these glucose sensors was collected using the iPro transmitters, which were better suited for in vivo use. Because of the timeline of receiving the iPro transmitters, a smaller sample of functional sensor data was collected (n < 10 sensors for each timepoint in non-diabetic and diabetic tissue). Out of a total of 16 sensors intended for the 1-month timepoint, five sensors continued to produce raw sensor signal at 28 days. The Enlite 2 sensors were intended for the 1-week timepoint only. In general, sensors were able to produce signal for longer than 3 weeks. Unfortunately, for the small sample size using iPro transmitters, a considerable amount of sensors failed before the 1-month timepoint. In previous studies using GST transmitters, 1-month sensor data was acquired, however these data were incorrectly calibrated and noisy.
Table 13: Sensor life for all sensor types.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic Sof</th>
<th>Diabetic Sof</th>
<th>Non-diabetic Enlite</th>
<th>Non-diabetic Enlite 2</th>
<th>Diabetic Enlite 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Sensors</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>3 day</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>1 month</td>
<td>5</td>
<td>7</td>
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<td></td>
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<td>Rat death</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensor life = 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Sensor life &lt; 1 wk</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sensor life &lt; 2 wk</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Sensor life &lt; 3 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensor life &lt; 4 wk</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td></td>
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<tr>
<td>3 day success rate</td>
<td></td>
<td></td>
<td>1/1 (100%)</td>
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<tr>
<td>7 day success rate</td>
<td></td>
<td></td>
<td></td>
<td>8/12 (67%)</td>
<td>8/12 (67%)</td>
</tr>
<tr>
<td>28 day success rate</td>
<td>1/5 (20%)</td>
<td>3/5 (60%)</td>
<td>1/6 (17%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
13.3.4.1: Sensitivity Analysis

Sensitivity to glucose (nA/mg/dl) was calculated for each functional sensor type as previously described. Figure 96 illustrates sensitivity to glucose over time for all sensor types. Enlite 2 sensors were only analyzed at the 1-week timepoint. For 1-month sensors, sensitivity to glucose decreased steadily over time for all sensors except for the non-diabetic Enlite sensor.

![Sensitivity to Glucose over Time](image)

**Figure 96**: Sensitivity to glucose over time for all functional sensors. Values are mean ± SEM.

Figure 97 illustrates the comparison in sensitivity between functional Sof and Enlite sensors in non-diabetic tissue. Over the first ten days of sensor life, the Enlite sensor had a greater sensitivity to glucose, with significance at Day 3 (p = 0.0001) and 1 week (p = 0.009). As the 1-month timepoint approaches, sensitivity to glucose gradually decreased for the Sof sensor. Interestingly, sensitivity to glucose remained relatively stable for the
Enlite sensor, decreasing at times, but eventually increasing towards the 1-month timepoint.

**Sensitivity - Non-diabetic Sensor Comparison**

Figure 97: Sensor comparison of sensitivity to glucose in non-diabetic tissue. Asterisk indicates $p < 0.05$ on days 3 and 7. Values are mean ± SEM.

Figure 98 illustrates differences in sensitivity to glucose for the Sof sensor in non-diabetic and diabetic tissue. No significant differences in sensitivity were observed at the 3-day, 1-week, or 1-month timepoints, however sensitivity to glucose appeared to be greater in non-diabetic tissue.
Figure 98: Comparison of Sof sensor sensitivity to glucose in non-diabetic and diabetic tissue. Values are mean ± SEM.

Sensitivity to glucose was also compared for the 1-week timepoint Enlite 2 sensors. As seen from Figure 99, one week was not a sufficient timeframe to observe any noticeable changes in sensitivity to glucose, however sensitivity was consistently greater in diabetic tissue.
Figure 99: Comparison of Enlite 2 sensor sensitivity to glucose in non-diabetic and diabetic tissue. Values are mean ± SEM.

13.3.4.2: Lag Time and MARD Analysis

To test the performance of the sensors, a series of insulin and glucose bolus challenges were administered. During these bolus events, many more blood glucose measurements are collected, allowing for determination of lag time and mean absolute relative deviation. These analyses were difficult to perform on the entirety of the sensor data as sensors, though correctly calibrated, were only calibrated twice a day twelve hours apart. These glucose sensors, when implanted in human patients, are typically calibrated 4-6 times daily.

Table 14 lists the frequency of “value editing” during bolus events for the different sensor types. As mentioned previously, the proprietary algorithms for each sensor have
logic to filter out sensor signal if certain conditions are met. These conditions can be seen in Proprietary Appendix C. Unfortunately, many sensors experienced edited values during the bolus events. Interestingly, sensor editing was a greater issue in non-diabetic tissues than in diabetic tissue. In particular, the Enlite and Enlite 2 implanted in non-diabetic tissue had significant instances of edited values.

**Table 14: Sensors with edited values during bolus events.**

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic Sof</th>
<th>Non-diabetic Enlite</th>
<th>Diabetic Sof</th>
<th>Non-diabetic Enlite 2</th>
<th>Diabetic Enlite 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bolus 1 (1 day)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensors with edited values</td>
<td>2 (33%)</td>
<td>5 (71%)</td>
<td>2 (29%)</td>
<td>7 (78%)</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>Total Sensors</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td><strong>Bolus 2 (1 week)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensors with edited values</td>
<td>3 (50%)</td>
<td>3 (60%)</td>
<td>2 (33%)</td>
<td>6 (86%)</td>
<td>2 (25%)</td>
</tr>
<tr>
<td>Total Sensors</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td><strong>Bolus 3 (2 week)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensors with edited values</td>
<td>6 (100%)</td>
<td>4 (80%)</td>
<td>4 (80%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Sensors</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bolus 4 (3 week)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensors with edited values</td>
<td>2 (66%)</td>
<td>4 (100%)</td>
<td>3 (75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Sensors</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lag time analysis was performed on sensors for bolus events without edited values. Lag time was calculated using the procedure described in the Methods section. In many cases, the sample size was too low to test for significance. Figure 100 illustrates differences in lag time among sensors. During the 1-day bolus, no significant differences
in lag time were seen among sensor types. Performance in non-diabetic and diabetic tissue was relatively identical. During the 1-week bolus, the diabetic Sof sensor had a significantly greater lag time than the diabetic Enlite 2 sensor (p = 0.010). Unfortunately, the sample sizes were too small to make any definitive conclusions concerning lag time over the life of the sensor, with only the diabetic Sof sensor having data for each bolus.

![Lag Time - Sensor Comparison](image)

**Figure 100**: Lag time comparison between all functional sensor types for bolus events. Asterisk (*) indicates p < 0.05. Bolus events without error bars were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

Lag time of sensors was also compared in non-diabetic and diabetic tissue as can be seen in Figure 101. No significant differences in lag time were observed during any bolus
event in non-diabetic and diabetic tissue. Additionally, lag time remained relatively stable over the lifetime of the sensor.

![Lag Time - Non-diabetic vs. Diabetic](image)

**Figure 101:** Non-diabetic vs. diabetic lag time comparison for all functional sensor types for bolus events. Bolus events without error bars were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

Finally, sensors were evaluated on their performance for each bolus event (Figure 102). Interestingly, lag time does appear to decrease over time for the non-diabetic and diabetic Sof sensors, though these results were not significant. Lag time for the non-diabetic Enlite sensor remained relatively constant over different bolus events. Finally, a significant decrease in lag time was observed between the 1-day and 1-week bolus for the diabetic Enlite 2 sensor (p = 0.005).
Figure 102: Comparison of bolus events over time for all functional sensor types. Asterisk (*) indicates p < 0.05. Bolus events without error bars were not evaluated for statistical significance due to low sample size. Values are mean ± SEM.

Similarly, MARD values were calculated for all bolus events without edited sensor values (Table 15). MARD was calculated for two glycemic ranges, BG < 120 mg/dl and BG > 120 mg/dl. In many cases, these values were identical for sensors implanted in diabetic tissues. For the Day-1 bolus, diabetic sensors had considerably lower MARD values than non-diabetic sensors. Additionally, for the non-diabetic sensors, MARD increased at blood glucose values greater than 120 mg/dl. For the 1-week bolus, MARD values for the non-diabetic Enlite and Enlite 2 sensors decreased considerably, while values for the non-diabetic Sof sensor remained constant. The diabetic Sof sensor experienced a significant increase in MARD (p = 0.016, denoted with *), to a value
similar to the non-diabetic Sof sensor. Conversely, MARD values for the diabetic Enlite 2 sensor decreased significantly from the 1-day to 1-week bolus ($p = 0.029$, denoted with $^\wedge$). For the non-diabetic Enlite sensor, MARD values again increased considerably for BG values greater than 120mg/dl, however MARD values for the non-diabetic Sof and Enlite 2 sensors decreased in this glycemic range. Insufficient data was available to make conclusions about later bolus events.

Table 15: MARD (%) values for all sensors for bolus events. All symbols ($^*$, $^\wedge$) indicate $p < 0.05$.

<table>
<thead>
<tr>
<th>Bolus 1</th>
<th>Non-diabetic Sof</th>
<th>Diabetic Sof</th>
<th>Non-diabetic Enlite</th>
<th>Non-diabetic Enlite 2</th>
<th>Diabetic Enlite 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARD (%)</td>
<td>32.3 ± 7.2</td>
<td>19.6 ± 4.1*</td>
<td>34.4</td>
<td>51.5</td>
<td>26.1 ± 3.4$^\wedge$</td>
</tr>
<tr>
<td>MARD &gt;120 mg/dl</td>
<td>43.4 ± 10.9</td>
<td>19.6 ± 4.1</td>
<td>50.1</td>
<td>60.6</td>
<td>26.1 ± 3.4</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Bolus 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARD</td>
<td>32.6 ± 9.1</td>
<td>39.7 ± 3.7*</td>
<td>22.2</td>
<td>25.5</td>
<td>14.6 ± 3.6$^\wedge$</td>
</tr>
<tr>
<td>MARD &gt;120 mg/dl</td>
<td>30.6 ± 8.1</td>
<td>39.7 ± 3.7</td>
<td>34.1</td>
<td>15.8</td>
<td>14.6 ± 3.6</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Bolus 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARD &gt;120 mg/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bolus 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARD</td>
<td>59.7</td>
<td>45.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARD &gt;120 mg/dl</td>
<td>65.3</td>
<td>20.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>1</td>
<td>1</td>
<td></td>
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</tbody>
</table>

13.3.5: Rat Hemoglobin A1c Assay

The HbA1c calibration curve was constructed by plotting the mean change in absorbance value for each calibrator on the Y-axis versus the corresponding HbA1c concentration (5.5% or 9.5%) on the X-axis (Figure 103). Rat HbA1c concentrations in the
blood samples were interpolated using the calibration curve and mean absorbance values for each sample.

![Calibration Curve HbA1c](image)

**Figure 103: Example calibration curve for determination of %HbA1c in rat whole blood.**

On Day 0, no significant differences were observed in %HbA1c among the 8 rats. The %HbA1c for rats set aside for streptozotocin injections was $5.717 \pm 0.432\%$ (mean ± SEM). The %HbA1c for non-diabetic rats was $5.266 \pm 1.207\%$. After blood was collected from all rats on Day 0, four rats received streptozotocin injections and became diabetic. Blood was then collected from the tail vein at 3 weeks and 7 weeks. Significant differences in %HbA1c were observed at both 3 and 7 weeks. %HbA1c increased to $8.472 \pm 0.409\%$ for the diabetic group at 3 weeks and remained constant at 7 weeks ($8.710 \pm 0.308\%$) (Figure 104).
A variety of factors exist that contribute to wound healing deficiencies in diabetics, including a diminished angiogenic response, decreased macrophage function, and altered collagen accumulation. All of these factors are particularly applicable in the case of implantable glucose sensors. Typically in healthy tissues, implantation of a foreign device triggers an immediate immune response that involves recruitment of inflammatory cells. As the foreign body response progresses, a dense collagen capsule forms around the device and microvessels regress. These events are deleterious to the functioning of percutaneously-implanted glucose sensors in healthy tissue, however little is known about the progression of implant healing in diabetic tissue. Three types
of percutaneously-implanted, amperometric glucose sensors were tested in non-diabetic and diabetic tissue. The Sof™ sensor is an older glucose sensor design comprised of a stiff, wire-like electrode covered with protective, plastic tubing. The Enlite™ and Enlite 2™ sensors are significantly thinner, shorter, and more flexible than the Sof™ sensor, with designs intended to address concerns that sensor micromotion promotes more aggressive inflammatory responses. The Enlite 2™ sensor differs from the Enlite™ sensor in that it consists of only a flexible electrode with no protective tubing. This study was therefore designed to understand and evaluate the tissue response to different sensor designs in non-diabetic and diabetic tissue. Tissue adjacent to implanted sensors was evaluated for collagen content and collagen density of any fibrous encapsulation, microvessel density, and inflammatory cell density. Sensor performance was also evaluated via analysis of sensitivity to glucose, lag time, and MARD.

13.4.1: Collagen Capsule Thickness and Collagen Density Index

Previous studies have shown that fibrous encapsulation inhibits diffusion of small analytes to implant surfaces\textsuperscript{146}. Additionally, lag in sensor response is generally attributed to greater resistance to mass transfer\textsuperscript{147}. The presence of a highly-resistive, avascular fibrous capsule results in decreased analyte diffusion to the sensor surface, which may affect glucose and oxygen levels at the sensor-tissue interface\textsuperscript{148}. All of these events may be deleterious to glucose sensor performance.
13.4.1.1: Histology

Representative images of Masson’s trichrome-stained tissue samples were used to visualize changes in collagen content over time. The beginning stages of collagen capsule formation can be seen at the 3-day timepoint for both percutaneous Sof and Enlite sensors in non-diabetic and diabetic tissue. Most evident from the histological images however, was the considerable increase in inflammatory cell density adjacent to sensors over time. Collagen capsule formation, while still present, particular in the case of the non-diabetic percutaneous Sof sensor, was seemingly overshadowed by the chronic inflammatory response. In many instances, collagen capsule formation occurred further removed from the sensor surface, encompassing the dense region of cells directly adjacent to the sensor. Infiltration of inflammatory cells likely affects the development of collagen capsule formation, as many sensors did not develop capsules if there was an aggressive inflammatory response.

When comparing the percutaneous Enlite and Enlite 2 sensors in non-diabetic tissue at 1-week, collagen capsule formation was more evident adjacent to the Enlite sensor. Collagen capsule formation was not evident adjacent to the non-diabetic Enlite 2 sensor, most likely because of the presence of a considerable number of inflammatory cells. The increased inflammation around the Enlite 2 sensor can likely be attributed to a more adverse tissue reaction to the bare electrode, which is partially covered in the Enlite
sensor. In diabetic tissue, percutaneously-implanted Enlite 2 sensors and fully-implanted Sof and Enlite sensors were compared to their counterparts at 1-week. Little inflammation and collagen capsule formation were seen from Masson’s trichrome-stained tissue samples of diabetic Enlite Per. and Enlite 2 Per. sensors, an indication of the delayed immune response and altered collagen formation in diabetics. Interestingly, the diabetic percutaneous Sof sensor had a considerable inflammatory response at 1-week, which can possibly be attributed to the size and stiffness of the sensor, as well as increased micromotion due to implant size. Subcutaneous sensors in diabetic tissue at 1-week had disorganized collagen formation and considerable inflammation, which may be influenced by excess hydrogen peroxide locally. Subcutaneous Enlite sensors in diabetic and non-diabetic tissue at 1 month varied in tissue response; in non-diabetic tissue, inflammation was considerably less and collagen development was better defined adjacent to the subcutaneous sensor, while the subcutaneous sensor in diabetic tissue was highly inflamed.

13.4.1.2: Collagen Capsule Thickness

Collagen capsule thickness was quantified for all sensors. Sensors that did not develop collagen capsules were assigned a thickness of 0. Thickness comparisons were made for both the total number of sensors and encapsulated sensors only. When comparing non-diabetic and diabetic tissue responses, no significant differences in collagen thickness were seen at any timepoint for total sensors or encapsulated sensors.
When comparing differences between sensors, significant differences in collagen thickness were observed for total and encapsulated sensors at early timepoints, but these differences dissipated by the 1-month timepoint. At 1 month, collagen capsule formation has likely stabilized and is then largely unaffected by factors such as sensor size or stiffness. At the 3-day timepoint, collagen thickness for the total percutaneous Sof sensors was significantly greater than that of percutaneous Enlite sensors in diabetic tissue, which can possibly be attributed to the greater size and stiffness of the Sof sensor. At the 1-week timepoint however, this difference was not observed. In fact, no significant differences in collagen thickness were observed among total sensors implanted in diabetic tissue at the 1-week timepoint. This result suggests that differences in sensor design or external mechanical stress (percutaneous vs. subcutaneous) are not significant enough to produce consequent differences in collagen encapsulation for total sensors. When only encapsulated sensors were considered however, significant differences were observed among sensors in diabetic tissue at the 1-week timepoint. Collagen thickness adjacent to percutaneous Enlite sensors was significantly greater than that of percutaneous Enlite 2 sensors in diabetic tissue, a result that may be explained by increased inflammatory cells adjacent to Enlite 2 sensors. Collagen thickness adjacent to percutaneous Enlite sensors was also significantly greater than that of fully-implanted, subcutaneous Enlite sensors, which could result from the
combination of external mechanical stress on the percutaneous sensors and excess hydrogen peroxide adjacent to subcutaneous sensors. In non-diabetic tissue at 1 week, percutaneous Enlite and Sof sensors had greater collagen encapsulation than percutaneous Enlite 2 sensors.

Few significant differences in collagen thickness were observed over time for any sensor. In general, collagen capsule thickness for all sensors remained stable or increased slightly at later timepoints. These results suggest that the foreign body response to implanted sensors equilibrates over time, with a balance established between collagen development and inflammatory cell activity.

13.4.1.3: Collagen Density Index

Collagen density index (CDI) measurements were taken from Masson’s trichrome-stained tissue samples of encapsulated sensors. When comparing sensors in non-diabetic and diabetic tissue, no significant differences in CDI were observed at any timepoint. These results seem to suggest that collagen density of capsules surrounding implanted sensors is largely unaffected by tissue type.

When sensor comparisons of collagen density were made, significant differences were observed at the 1-week timepoint. In non-diabetic tissue, the percutaneous Sof sensors had a significantly greater CDI than the percutaneous Enlite 2 sensors. Likely collagen
encapsulation adjacent to the Sof sensors is denser due to a higher presence of inflammatory cells adjacent to the Enlite 2 sensors. In diabetic tissue, the percutaneous Enlite sensors had a significantly greater CDI than the percutaneous Enlite 2 sensors, which may also be the result of a greater inflammatory response caused by tissue reaction to the bare electrode of the Enlite 2 sensor. At the 1-month timepoint, which does not include Enlite 2 sensors, no significant differences in CDI were observed among sensor types.

When changes in collagen density index for encapsulated sensors were observed over time, few significant differences were seen among the sensor types. CDI remained relatively constant as the immune response progresses for all sensor types except for the non-diabetic percutaneous Sof sensor. CDI adjacent to this sensor remained relatively unchanged from the 3-day to 1-week timepoints and decreased significantly at the 1-month timepoint. Although it is difficult to determine the exact cause of this decrease in CDI, remodeling of collagen does typically occur weeks to months after initial wounding/implantation.

13.4.2: Inflammatory Cell Density

Glucose sensor performance and lifetime may be adversely affected by the migration of inflammatory cells to the implant site. During the foreign body response, nearby inflammatory cells become activated, resulting in the decrease of local pH and the
production of superoxide and peroxide. These compounds have been linked to poor
sensor performance\textsuperscript{148, 175, 176}. In addition, these cells increase consumption of local
glucose levels, resulting in potentially inaccurate blood glucose readings by the sensor.
Inflammatory cell density was evaluated in three zones surrounding the sensor using
Hoechst 33342-stained histology sections.

13.4.2.1: Histology

For visualization and quantification of inflammatory cell density, tissue samples were
stained with hematoxylin and eosin (H&E) and Hoechst 33342. Both stains target cell
nuclei; changes in stain over time can be attributed to the migration of inflammatory
cells as native stromal cells do not proliferate very rapidly. Histological images confirm
much of what was seen in Masson’s trichrome-stained tissue samples. Inflammatory cell
density was relatively sparse at the 3-day timepoint, but increased considerably over
time as the tissue adjacent to sensors became more and more inflamed. These images
indicate the presence of percutaneously-implanted sensors resulted in a chronic
inflammatory response where inflammatory cells persist and collagen capsule formation
stabilizes over time.

Similarly to the Masson’s trichrome-stained images, H&E- and Hoechst 33342-stained
tissue samples of percutaneous Enlite 2 sensors in non-diabetic tissue showed greater
inflammatory cell density in comparison to percutaneous Enlite sensors at 1 week.
Again, this result is likely caused by the tissue reaction to the bare electrode. In diabetic tissue, considerable inflammation was seen adjacent to percutaneous and subcutaneous Sof sensors at 1 week. This inflammation was concentrated largely adjacent to the sensing electrode. Inflammatory cell density appeared greater adjacent to percutaneous Enlite 2 sensors and subcutaneous Enlite sensors in comparison to the percutaneous Enlite sensors in diabetic tissue.

At the 1-month timepoint, histological images show considerable inflammation adjacent to subcutaneous Enlite sensors in both non-diabetic and diabetic tissue, which is likely due to the buildup of excess hydrogen peroxide.

13.4.2.2: Inflammatory Cell Density

Inflammatory cell density was quantified using the previously-described protocol. When comparing sensors in non-diabetic and diabetic tissue, no significant differences in inflammatory cell density were observed at the 3-day timepoint in the 0-50 μm distance zone. In all zones, inflammation surrounding sensors implanted in diabetic tissue was somewhat lower, though these results were not significant. In the most proximal zone to the sensor, inflammatory cell density was greater adjacent to percutaneous Enlite sensors in comparison to percutaneous Sof sensors in both tissue types, though these results were also not significant. Interestingly, in the 100-200 μm zone, inflammatory cell density adjacent to percutaneous Enlite sensors was
significantly greater in diabetic tissue in comparison to non-diabetic tissue. At the 1-week timepoint, no significant differences in inflammatory cell density were observed when sensors were compared in non-diabetic and diabetic tissues. Inflammatory cell density was relatively equal for all sensor types, but was not consistently greater in one tissue type. Similar results were observed at the 1-month timepoint. These results indicate that the inflammatory response in diabetic tissue is comparable to that in non-diabetic tissue and not significantly diminished as hypothesized.

When comparisons among sensors were made, inflammatory cell density adjacent to percutaneous Enlite sensors was greater than adjacent to percutaneous Sof sensors in all zones and tissue types at the 3-day timepoint. In the 0-50 μm zone, inflammation surrounding the percutaneous Enlite sensor was significantly greater than that surrounding the percutaneous Sof sensor in non-diabetic tissue, a result that was somewhat unexpected due to the slimmer, more flexible design of the Enlite sensor. One possible explanation however is that the slimmer sensor design increases the ratio of sensing electrode to plastic housing, a factor that could outweigh the benefits of smaller size and flexibility. Inflammatory cell density has been shown to be greater directly adjacent to the sensing electrode. In the 100-200 μm zone, the inflammatory cell density adjacent to the percutaneous Enlite sensors was significantly greater than that of the percutaneous Sof sensors in diabetic tissue. At the 1-week timepoint, the
subcutaneous Sof sensors had a significantly greater inflammatory cell density than the percutaneous Enlite sensors in the 50-100 μm zone of diabetic tissue. However, too many variables differ between the two sensor types to determine the precise cause of this difference. No other significant differences in inflammatory cell density were observed among sensor types at the 1-week timepoint. At the 1-month timepoint, inflammation adjacent to percutaneous Enlite sensors was significantly greater than that of percutaneous Sof sensors in the 100-200 μm zone of non-diabetic tissue. This result was also observed in all other distance zones and tissue types, though the differences are not significant. Finally, the inflammatory cell density adjacent to percutaneous Enlite sensors was greater than that of subcutaneous Enlite sensors in diabetic tissue, which can most likely be attributed to external mechanical stress from the attached sensor housing and transmitter.

When changes in inflammation were observed over time, inflammatory cell density increased steadily for most sensor types in the 0-50 μm zone. These increases were significant in the case of the non-diabetic and diabetic percutaneous Sof sensors and seem to depict the recruitment of inflammatory cells as the immune response shifts from the acute to the chronic phase. The inflammatory cell density adjacent to non-diabetic percutaneous Enlite sensors was considerably high at the 3-day timepoint and remained relatively constant over time. This behavior was also observed for the diabetic
subcutaneous Enlite sensors in the 0-50 \( \mu \text{m} \) zone. In the 50-100 \( \mu \text{m} \) zone, inflammatory cell density increased over time for all percutaneously-implanted, functional sensors.

The inflammatory cell density adjacent to subcutaneous Enlite sensors remained constant over time. Similar responses are observed in the 100-200 \( \mu \text{m} \) zone, however inflammatory cell density remains stable for the diabetic percutaneous Enlite sensors.

These results emphasize the continual recruitment of inflammatory cells over time. At the 1-month timepoint, inflammatory cell density for all sensor types was either stable or increasing, an indication that the immune response is in a chronic state.

Prior to inflammatory cell density analysis, it was hypothesized that the slimmer, more flexible designs of the Enlite and Enlite 2 sensors would reduce implant micromotion and promote a less aggressive inflammatory response. On the contrary, percutaneous Enlite and Enlite 2 sensors generally produced a greater inflammatory response in comparison with Sof sensors. These results seem to suggest that implant micromotion plays a lesser role in stimulating the immune response than other factors. In addition, development of the Vetrap animal wrapping protocol may have sufficiently stabilized external hardware to the point where differences in inflammatory response are due to the sensors themselves. Indeed, inflammatory cell density had been shown to be greatest directly adjacent to the sensing electrode of glucose sensors. The Enlite and
Enlite 2 sensors, while smaller and more flexible, have more of the sensing electrode exposed to tissue, which may explain the greater inflammatory response.

13.4.2.3: Macrophage Immunohistochemistry

The CD68 macrophage staining was performed on a small subset of functional sensors only. The CD68-stained images show variable responses over time for the different sensor types. In non-diabetic tissue, macrophage density adjacent to percutaneous Sof sensors appears to increase gradually over time. On the other hand, macrophage density remained relatively constant over time adjacent to percutaneous Enlite sensors in non-diabetic tissue. In diabetic tissue, macrophage density adjacent to Sof sensors remained relatively constant, but increased considerably adjacent to Enlite sensors. In both non-diabetic and diabetic tissue, macrophage concentration appears most dense adjacent to the percutaneous Enlite sensors. These observations are consistent with those made concerning total inflammatory cell density.

At the 1-week timepoint, macrophage density adjacent to percutaneous Enlite 2 sensors was compared to that adjacent to percutaneous Enlite sensors. In both non-diabetic and diabetic tissue, macrophage density adjacent to Enlite 2 sensors appears greater than that adjacent to Enlite sensors. This increased inflammation is likely the result of tissue reaction to the sensing electrode.
13.4.2.4: Macrophage Density

Macrophage density was quantified using the previously-described protocol. Macrophage counts were graphed alongside total inflammatory cell counts as a way to visually assess the foreign body response. At the 3-day timepoint, percutaneous Enlite sensors in both non-diabetic and diabetic tissue had greater macrophage and inflammatory cell density in comparison to percutaneous Sof sensors in the 0-50 μm zone. Macrophage and total inflammatory cell counts decreased for all sensor types at zones further removed from the sensors. At the 1-week timepoint, macrophage density was relatively equal for all functional sensor types and tissues in the 0-50 μm zone. In more distant zones, the diabetic percutaneous Enlite sensors had the greatest macrophage density of all sensors. Interestingly, macrophages make up a larger portion of the total inflammatory response for the non-diabetic Sof sensor than for all other sensors. This observation could result from a healthy immune response to a considerably larger implant. At the 1-month timepoint, macrophage density adjacent to non-diabetic sensors was greater than that of diabetic sensors in the 0-50 μm zone. The macrophage density of non-diabetic percutaneous Enlite sensors was the greatest of all sensors at all distance zones at 1 month, which again is consistent with previous findings.
Macrophage counts were also expressed as ratios for easier visual comparison. At the 3-day timepoint, the ratio of macrophages to total cells was relatively similar for all sensor types. At the 1-week timepoint, the ratio of macrophages to total cells increased considerably for the non-diabetic Sof sensor for all distance zones, however considerable variability is present. In general, ratios were fairly consistent among sensor types, with slightly fewer macrophages/total cells present in the 0-50 µm zone of diabetic tissue in comparison to non-diabetic tissue. At the 1-month timepoint, the non-diabetic Enlite sensors had the greatest ratio of macrophages to total cells, however this result was not significant. In general, ratios were fairly consistent among sensors at all distance zones, which seems to suggest stabilization of the foreign body response.

When comparisons were made among sensors, macrophage density adjacent to percutaneous Enlite sensors was greater than that of Sof sensors in all distance zones at the 3-day timepoint. At the 1-week timepoint, all sensor types had fairly similar macrophage densities in the 0-50 µm zone. In more distant zones, percutaneous Enlite sensors exhibited greater macrophage densities. Finally, at the 1-month timepoint, the percutaneous Enlite sensors generally had the greatest macrophage densities at all distance zones, though considerable variability in the counts exists.
Macrophage density was also compared for each sensor in non-diabetic and diabetic tissue. At the 3-day timepoint, macrophage densities were relatively similar for both sensor types in the 0-50 μm zone. In further distance zones, macrophage densities remained similar for the percutaneous Sof sensors in non-diabetic and diabetic tissue. In contrast, macrophage density in non-diabetic tissue was considerably greater than that in diabetic tissue in further distance zones for the percutaneous Enlite sensors. At the 1-week timepoint, macrophage densities for the percutaneous Sof and Enlite 2 sensors were essentially equal in non-diabetic and diabetic tissue for all distance zones. In the 0-50 μm zone, the macrophage density adjacent to the non-diabetic Enlite sensors was greater than that of diabetic Enlite sensors. Interestingly, the macrophage density adjacent to non-diabetic Enlite sensors decreased with increasing distance from the sensor while the macrophage density adjacent to diabetic Enlite sensors remained constant. In the 100-200 μm zone, the diabetic Enlite sensors had a greater macrophage density, which may suggest a delayed or sustained inflammatory response. At the 1-month timepoint, the non-diabetic Enlite sensors had a considerably greater macrophage density than the diabetic Enlite sensors for all distance zones, though there is much variability in the data. No considerable differences in macrophage density were noted in non-diabetic and diabetic tissue for the percutaneous Sof sensors.
Lastly, macrophage density was analyzed over the different timepoints. Macrophage density for the non-diabetic Sof sensors increased from the 3-day to 1-week timepoints and remained constant from the 1-week to 1-month timepoints in the 0-50 μm zone. Both non-diabetic Enlite sensors and diabetic Sof sensors had stable macrophage densities over time, while macrophage density for the diabetic Enlite sensor decreased over time for the 0-50 μm zone. In general, these results demonstrate early macrophage activation and recruitment and a sustained inflammatory response during the chronic inflammatory phase. In the 50-100 μm zone, macrophage density was relatively constant over time for all sensors for all distance zones. Macrophage density adjacent to the non-diabetic Enlite sensors was considerably greater in comparison to all other sensors. Similar responses were observed over time in the 100-200 μm zone, though relative numbers of macrophages were lower than in the 50-100 μm zone. Interestingly, macrophage density adjacent to diabetic Enlite sensors increased over time in the 100-200 μm zone despite decreasing over time in the 0-50 μm zone.

13.4.3: Microvessel Density

The presence of microvessels in the tissue adjacent to implanted glucose sensors is essential to the delivery of glucose and accurate sensor response. Unfortunately, collagen capsule formation generally leads to microvessel regression, which means that glucose must diffuse across a greater distance in order to reach the sensor. This results in increased sensor lag times and sensor glucose readings that are not reflective of actual
blood glucose levels. In addition, the process of angiogenesis is generally impaired in diabetic tissue, which could be even more problematic for glucose sensor performance in diabetic tissue. Microvessel density in zones adjacent to implanted glucose sensors were thus quantified in CD31-stained tissue samples.

13.4.3.1: Microvessel Immunohistochemistry

CD31-stained tissue samples were used to assess changes in microvessel density over time. Judging from CD31 images, microvessel density appears to decrease over time for long-term diabetic percutaneous Sof and Enlite sensors. This microvessel regression may be influenced by slight increases or stabilization of collagen capsule formation surrounding the sensors. In non-diabetic tissue, microvessel density appears to increase adjacent to the percutaneous Sof sensors and decrease slightly for the percutaneous Enlite sensors. The decrease in microvessel density over time for the non-diabetic Enlite sensors may be explained by the considerable inflammation surrounding these sensors, which may negate the positive effect of the release of proangiogenic growth factors.

At the 1-week timepoint, percutaneous Enlite 2 and Enlite sensors had similar microvessel densities in non-diabetic tissue as seen from CD31-stained images. In diabetic tissue, the percutaneous Enlite 2 sensor appears to have a slightly lower microvessel density in comparison to the percutaneous Enlite sensor at 1 week. Interestingly, subcutaneous Sof and Enlite sensors appear to have greater microvessel
densities than their percutaneous counterparts in diabetic tissue at 1 week. Similarly, subcutaneous Enlite sensors appear to have greater microvessel densities than their percutaneous counterparts in non-diabetic and diabetic tissue at the 1-month timepoint. These results are interesting as collagen thicknesses and inflammatory cell densities are relatively similar for subcutaneous and percutaneous sensors at these timepoints. The increase in microvessel density may possibly be attributed to lack of external mechanical stress on the subcutaneous sensors.

13.4.3.2: Microvessel Density

Microvessel density was quantified using the previously-described protocol. When microvessel density was compared in non-diabetic and diabetic tissue, no differences were observed for percutaneous Sof sensors in any distance zone at the 3-day timepoint. 3-day vasculature was relatively equal for the percutaneous Sof sensors, at levels somewhat less than native tissue. In contrast, microvessel density adjacent to non-diabetic percutaneous Enlite sensors was considerably greater than that in diabetic tissue and relatively equal to native tissue microvessel density. This result is notable because non-diabetic percutaneous Enlite sensors also have the greatest inflammatory cell density. Clearly, a balance exists between inflammation and angiogenesis. Macrophages, along with other cell types, release proangiogenic growth factors, which explains the response observed for non-diabetic percutaneous Enlite sensors. At the 1-week timepoint, the microvessel density adjacent to non-diabetic percutaneous Enlite
sensors was significantly greater than that of diabetic percutaneous Enlite sensors at all distance zones. This result was expected since angiogenesis is generally deficient in diabetic tissue. Vasculature adjacent to other non-diabetic sensors was generally greater in comparison to diabetic counterparts, however the difference was not as pronounced as for the percutaneous Enlite sensor. At the 1-month timepoint, vascularity for all sensors in the 0-50 μm zone was considerably less than that of native tissue except for the diabetic percutaneous Sof sensors. Microvessel density adjacent to the diabetic percutaneous Sof sensors was considerably greater than that of non-diabetic Sof sensors, though considerable variability is present. In more distant zones, vasculature for all percutaneous sensors equilibrated to native tissue levels. In the case of the subcutaneous Enlite sensors, vascularity was greater in non-diabetic tissues, reaching significance in the 100-200 μm zone.

When comparisons among sensor types were made, percutaneous Enlite sensors had considerably greater microvessel density than other sensor types for all distance zones in non-diabetic tissue, at levels similar to native tissue at the 3-day timepoint. In diabetic tissue, vascularity adjacent to percutaneous Enlite sensors was still the greatest of the sensor types, however this difference was less pronounced. At the 3-day timepoint, subcutaneous Enlite sensors in diabetic tissue had considerably less vascularity than either percutaneous sensor, which may be the result of excess hydrogen peroxide in
local tissue. At the 1-week timepoint, no significant differences in microvessel density were observed among sensor types in non-diabetic tissues. In diabetic tissue, percutaneous Enlite sensors exhibited a marked decrease in vascularity and had the lowest microvessel density of all sensor types in all distance zones. This response was potentially the result of greater inflammation adjacent to these sensors, however it is important to note that vasculature adjacent to non-diabetic percutaneous Enlite sensors does not appear to be affected by considerable inflammation. It is possible that the inflammation seen in the case of diabetic percutaneous Enlite sensors was sufficient to upset the balance between inflammation and angiogenesis. In confirmation with CD31-stained images, vascularity of subcutaneous sensors was greater (at times significantly) than that of percutaneous counterparts in diabetic tissue. This is likely the result of the absence of external mechanical stress on the sensor. Also at the 1-week timepoint, vascularity adjacent to diabetic percutaneous Enlite 2 sensors was greater than that of percutaneous Enlite sensors for all distance zones. This response is again likely due to proangiogenic growth factor release by greater numbers of inflammatory cells. Finally, microvessel density adjacent to percutaneous Sof sensors was significantly greater than that of percutaneous Enlite sensors at all distance zones in diabetic tissue, which is likely the result of reduced inflammation adjacent to the sensor. At the 1-month timepoint, no significant differences in microvessel density were observed among any sensor types. In non-diabetic tissue, vasculature adjacent to the subcutaneous Enlite sensor was greater.
than that of percutaneous Enlite sensors, a result that is consistent with CD31-stained images. In non-diabetic tissue, vasculature was considerably lower than native tissue for all sensor types in the 0-50 μm zone, but increased to native tissue levels at more distant zones. Vasculature adjacent to the diabetic percutaneous Sof sensors remained consistently at levels equal to native tissue for all distance zones. In addition, vasculature adjacent to diabetic percutaneous Enlite sensors was less than that of native tissue in the 0-50 μm zone, but increased to native levels at further zones. These results seem to suggest that while angiogenesis is generally deficient in diabetic tissue, given sufficient time, microvessel formation can recover in tissue adjacent to implanted devices.

When changes in vasculature were observed over time, microvessel density adjacent to non-diabetic percutaneous Sof sensors and diabetic percutaneous Enlite sensors remained relatively constant over time at levels somewhat below that of native tissue in the 0-50 μm zone. In non-diabetic tissue, microvessel density adjacent to percutaneous Enlite sensors began at levels equal to native tissue at the 3-day time point, but decreased and stabilized over time. This vessel regression is likely influenced by increased and persistent inflammation at the site of these sensors. Also in the 0-50 μm zone, vascularity adjacent to percutaneous Sof sensors and subcutaneous Enlite sensors increased over time in diabetic tissue, which seems to suggest a delayed angiogenic
response. In the 50-100 μm zone, microvessel density increased or remained stable over time for all sensor types. Likewise, vascularity increased or remained stable over time in the 100-200 μm zone.

13.4.4: Sensor Performance

The normal tissue response to percutaneously-implanted, functional glucose sensors can adversely affect sensor performance, accuracy, and lifetime as previously mentioned. However, since these glucose sensors were designed for use in diabetic patients, understanding how the diabetic tissue response affected sensor performance was crucial. In addition to histological endpoints, sensor performance was evaluated in non-diabetic and diabetic tissue. Three types of functional glucose sensors designed by Medtronic, Inc. (Sof™, Enlite™, Enlite 2™) were implanted percutaneously in non-diabetic and diabetic rat dorsum. The sensors were calibrated with a blood glucose measurement at least twice daily no more than 12 hours apart. Continuous raw sensor signal was collected over the lifetime of the sensors using iPro transmitters connected externally to the glucose sensors. These raw data files, along with all blood calibration measurements, were sent for processing by Medtronic, Inc. Proprietary algorithms specific to each sensor type were then used to compute sensor glucose (SG) values that could be compared to actual blood glucose measurements.
Processed data files provided by Medtronic, Inc. contained computed sensor glucose (SG) values paired with each blood calibration measurement and blood reading during bolus events. Previous studies involving Medtronic® glucose sensors have shown a linear relationship between sensor glucose values and glucose values in solution in vitro. To determine if such a relationship existed between SG and BG values in vivo, linear regression was performed on the paired data sets for each functional sensor type. This analysis produced r² values that not consistent with a linear relationship (r² < 0.6 for all sensor types). Therefore, the proprietary algorithms utilized by Medtronic, Inc. were essential to proper calibration and processing of glucose sensor data.

It is important to note that Medtronic® glucose sensors were designed for use in the blood glucose range of 40-400 mg/dl. For this reason, the proprietary algorithms often do not have logic to account for blood glucose ranges greater than 400 mg/dl as was the case for the Enlite sensor. Blood glucose ranges for the diabetic animals often exceeded 400 mg/dl. Therefore, Enlite sensors implanted in diabetic tissue were excluded in sensor performance analyses. Additionally, it is important to note that these sensors were implanted in animals with uncontrolled diabetes. Sensor response may be different in this uncontrolled model and results should not be projected to sensor function in humans, as humans typically medicate with insulin to prevent hyperglycemia.
Sensor life was determined for all sensor types. Only five out of twenty-one sensors connected to iPro transmitters survived to the 1-month timepoint. This low survival rate was partly due to the small sample size of sensors used with the iPro transmitters. Many sensors connected to GST transmitters survived to the 1-month timepoint, however these sensor data were not used for performance evaluation due to considerable background noise inherent with the GST transmitters and improper calibration of sensors. Shorter timepoints were generally not an issue for any sensor type. For example, Enlite 2 sensors had a 67% survival rate for the 1-week timepoint. Most long-term sensors failed between the 3rd and 4th weeks. Survival rate was greater for long-term sensors implanted in diabetic tissue. A possible explanation for this is the difference in health between diabetic and non-diabetic rats. Non-diabetic rats tended to be larger and more robust; oftentimes this greater activity led to more movement and frequent rat jacket changes. This extra mechanical stress most likely adversely affected sensor lifetimes.

13.4.4.1: Sensitivity Analysis

Sensitivity to glucose (nA/mg/dl) was computed for all sensor types over time. Sensitivity to glucose decreased over time for all long-term sensors except for the non-diabetic Enlite sensor. This result is interesting because the non-diabetic sensor had decreased microvessel density over time in addition to increased inflammatory cell
density. Short-term functional Enlite 2 sensors had no significant changes in sensitivity over time.

When comparing functional sensors implanted in non-diabetic tissue, the Enlite sensor had a greater sensitivity to glucose for the first 10 days, i.e. the acute inflammatory response. Sensitivity to glucose was then relatively equal for Sof and Enlite sensors for the 10-20 day time frame. Finally, sensitivity to glucose increased for the non-diabetic Enlite and decreased for the non-diabetic Sof sensor after 3 weeks. At days 3 and 7, the non-diabetic Enlite sensor had a significantly greater sensitivity to glucose than the Sof sensor. This may be a result of the greater vascularity adjacent to the non-diabetic Enlite sensor at early timepoints, which seems to balance the greater inflammatory cell density. Vascularity adjacent to the non-diabetic Enlite sensor decreased over time, but remained relatively stable between the 1-week and 1-month timepoints. The non-diabetic Sof sensor had a lesser inflammatory cell density than the non-diabetic Enlite sensor, but stable vascularity over time. These factors seem responsible for the relatively constant sensitivity shown by the non-diabetic Sof sensor.

When comparing sensor performance in non-diabetic and diabetic tissue, sensitivity to glucose for the Sof sensor was relatively equal, but generally higher in the non-diabetic Sof sensor. This could possibly be the result of over-saturation of glucose in diabetic
tissue. Conversely however, sensitivity to glucose was greater for the diabetic Enlite 2 sensor in comparison with the non-diabetic Enlite 2 sensor. However, the short timepoint of the Enlite 2 sensor may not have been sufficient to fully understand sensitivity behavior over time. Interesting to note is that oftentimes, no significant differences were observed for most parameters (inflammatory cell density, collagen thickness, microvessel density, sensitivity) when comparing non-diabetic and diabetic tissue. The foreign body response in diabetic tissue may be diminished, but not nearly to the extent as was originally hypothesized.

13.4.4.2: Lag Time and MARD Analysis

During the lifetime of each functional sensor type, sensor performance was frequently tested by the administration of insulin and glucose bolus challenges. These challenges allowed for the calculation of lag time and mean absolute relative deviation (MARD) of sensors. Lag time was calculated using the previously-described protocol. Briefly, for each raw sensor signal paired with a blood calibration measurement, the next five signal measurements were obtained. These signals were then used to compute associated sensor glucose (SG) values. By comparing these computed values to the initial blood glucose measurement, lag can be calculated in 5-minute increments. Unfortunately, several sensors experienced “value editing” during the bolus events. This editing can be caused by a number of conditions that are included in Proprietary Appendix C. The proprietary algorithms for each sensor have logic to filter out sensor signal in the case of
these conditions. Lag time and MARD were calculated for bolus events without edited values only. Table 14 lists the frequency of “value editing” during bolus events for each sensor type. Sensor editing was a much greater issue in non-diabetic tissue. In particular, the Enlite and Enlite 2 sensors implanted in non-diabetic tissue had significant instances of edited values (upwards of 60%). Poor performance of sensors in non-diabetic tissue is likely the result of more rapid changes in blood glucose levels during bolus events. Because rats were normoglycemic, infusion of insulin and glucose resulted in immediate, unphysiologic changes in blood glucose levels. These rapid changes were thus viewed as erroneous by the glucose sensor and filtered. In contrast, high levels of blood glucose present in diabetic rats may have resulted in less responsiveness to insulin and glucose challenges and therefore slower changes in sensor signal. Additionally, the administration of boluses intravenously most likely contributed to the frequency of value editing. IV injection had initially been employed because faster changes in blood glucose result in sharper, more well-defined peaks with which to evaluate lag time. Additionally, the use of IV injections decreases the amounts of insulin and glucose needed to produce changes in blood glucose levels as well as decreases the amount of time necessary to complete a bolus event. Unfortunately, rapid changes in blood glucose levels resulting from IV injection also most likely resulted in much of the observed sensor editing.
Lag time was compared among sensor types for each bolus event. Sample size for 2- and 3-week boluses was too few to make definitive conclusions concerning lag time over the life of the sensor. Lag time for the Day 1 bolus was relatively equal for all functional sensor types in diabetic tissue. In non-diabetic tissue, the percutaneous Sof sensor had a slightly longer lag time than the other sensors, however this result could not be tested for significance. For the 1-week bolus, lag time for the percutaneous Sof sensor in diabetic tissue was significantly greater than that of the Enlite 2 sensor. This may be the result of greater collagen capsule formation surrounding the Sof sensor at this timepoint. In non-diabetic tissue, lag time between Sof and Enlite sensors were relatively equal, while the Enlite 2 had a considerably longer lag time. Again, conclusions are difficult to make with the small sample size. When sensors were compared in non-diabetic and diabetic tissue, no significant differences in lag time were observed. For the percutaneous Sof sensor, lag time in non-diabetic tissue was greater for the Day 1 bolus, but lower for the 1-week bolus. In addition, lag time in each tissue type was relatively constant over the bolus events. Lag time for each sensor type was also analyzed over time to see if differences existed between bolus events. For the non-diabetic Sof sensor, lag time appears to decrease over time. Similarly, lag time for the diabetic Enlite 2 sensor decreased significantly from the Day 1 to 1-week bolus. Lag time for the Day 1 bolus is likely affected by tissue trauma resulting from the implantation of the sensor. Lag time for the non-diabetic Enlite sensors remained relatively constant over time,
though a decrease does occur between the Day 1 and 1-week boluses. Lag time for the diabetic Sof sensor decreased slightly over time. Lastly, in non-diabetic tissue, lag time for the Enlite 2 sensor increased from the Day 1 to 1-week boluses. In general however, lag times were fairly consistent among tissue types and sensor types over the course of the bolus events.

Calculation of mean absolute relative difference (MARD) is a traditional measure for the assessment of numerical accuracy of a glucose sensor. Numerical accuracy is defined as the closeness between continuous glucose monitoring (CGM) readings and corresponding in time reference blood glucose (BG) measurements\textsuperscript{177, 178}. MARD is calculated as the relative (in %) deviation of a sensor from reference. Other traditional means for calculating numerical accuracy are mean absolute difference (MAD) and ISO criteria. MAD gives an indication of the propensity of a particular continuous glucose sensor to read high or low compared to reference. Alternatively, ISO criteria are based on the percentage of sensor readings within 15 mg/dl from reference when the reference BG \( \leq 75 \) mg/dl, or within 20\% from reference when the reference BG > 75 mg/dl. These measures of numerical accuracy are useful in determining strict performance of glucose sensors, however they are very dependent on sample size and provide little clinical information. Error grid analysis was developed to evaluate clinical accuracy in order to assess the impact of sensor errors on treatment decisions based on CGM output\textsuperscript{177, 179}. 

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Specifically, the Clarke error grid consists of five regions in which a scatterplot of a reference glucose meter and an evaluated glucose meter is overlaid. Region A contains values within 20% of the reference sensor. Region B consists of values with a greater than 20% difference, but would not lead to inappropriate treatment. Region C contains points leading to unnecessary treatment, while Regions D and E represent points of significant sensor failure and treatment outcomes. The Clarke error grid has become widely accepted as a means to evaluate glucose sensor accuracy, however has been met with some criticism that the boundaries of the Regions are somewhat arbitrary.

MARD values were calculated for all sensors during bolus events as sufficient blood glucose measurements were available. MARD values for blood glucose levels above 120 mg/dl were calculated as to determine if sensor accuracy differed in normoglycemic and hyperglycemic conditions. For the Day 1 bolus, diabetic sensors had considerably lower MARD values than non-diabetic sensors. It is possible that despite uncontrolled hyperglycemia, diabetic rats have less variability in blood glucose levels due to impaired insulin secretion. In addition, it is possible that tissue trauma from sensor implantation has a greater effect on Day 1 sensor performance in non-diabetic tissue than in diabetic tissue. MARD values tended to increase at BG > 120 mg/dl for non-diabetic sensors at the Day 1 bolus, which would seem to suggest that these sensors are less accurate at higher blood glucose levels. However, sensors implanted in diabetic tissue actually
have lower MARD values for much higher BG levels for the Day 1 bolus. For the 1-week bolus, MARD values for the non-diabetic Enlite and Enlite 2 sensors decreased considerably, while values for the non-diabetic Sof sensor remained constant. The observed decrease could be a result of recovery from tissue trauma and stabilization of the sensor in adjacent tissue. The diabetic Sof sensor experienced a significant increase in MARD from the Day 1 to 1-week bolus even though lag time decreased somewhat. This could be the result of increased inflammatory cell density for the diabetic Sof sensor during this timepoint. MARD for the diabetic Enlite 2 sensor decreased significantly from the Day 1 to 1-week bolus, which mirrors the significant decrease in lag time. For the non-diabetic Enlite sensor, MARD values again increased considerably for BG > 120 mg/dl, however MARD values for the non-diabetic Sof and Enlite 2 sensors decreased in this glycemic range. In general, at the 1-week bolus, Sof sensors and Enlite 2 sensors had similar MARD values in non-diabetic and diabetic tissue, suggesting that sensor performance is not hindered by hyperglycemia. These results are reassuring and provide confidence that glucose sensors can function well in diabetic patients for at least short-term durations. Unfortunately, insufficient data was available to make conclusions about later bolus events.

13.4.5: Rat Hemoglobin A1c Assay

Hemoglobin A1c is an important test recommended for patients with diabetes every 2-3 months as part of the patient’s diabetes management program. Hyperglycemia, a
condition of diabetes, results in excess glucose in the blood. Glycohemoglobin is produced by non-enzymatic addition of glucose to amino groups in hemoglobin. HbA1c refers to glucose-modified hemoglobin A (HbA), specifically at N-terminal valine residues of hemoglobin beta chains. An HbA1c test is used both as an index of mean glycemia and as a measure of risk for the development of diabetes complications.

Measuring the percentage of HbA1c in the blood allows for the assessment of blood glucose levels of a certain timespan (typically 2-3 months). In healthy individuals, typical HbA1c ranges from 4-6%. The American Diabetes Association recommends an HbA1c under 7%.

Rat hemoglobin A1c kit from Crystal Chem, Inc. (Downers Grove, IL) was used for the assessment of HbA1c in diabetic and non-diabetic rat whole blood in order to further validate our diabetic model. A total of 8 rats were used for the assessment. On day 0, prior to any streptozotocin injections, whole blood was collected via tail vein from each rat. HbA1c was not significantly different among the rats and was within the typical healthy range of 4-6%. Immediately after blood collection on day 0, the rats were separated into two groups. The diabetic group then received sufficient STZ injections to develop Type 1 diabetes. Whole blood was again collected from all rats at 3 and 7 weeks. At 3 weeks, a significant difference in HbA1c was seen between the non-diabetic and diabetic groups. HbA1c for the diabetic group was greater than 7%, clearly
indicative of a diabetic state. HbA1c for the non-diabetic group remained in the healthy range mentioned previously. These results further validate our diabetic model. Our diabetic rats, in addition to hyperglycemia, develop immune deficiencies and increased levels of glycated hemoglobin as the disease progresses.

**13.4.6: Summary**

**Collagen Thickness and Collagen Density Index (CDI):**

- No significant differences in collagen thickness were observed over time between non-diabetic and diabetic tissue for any sensor type.

- Significant differences in collagen thickness among sensors were only seen at early timepoints. Long-term collagen development was unaffected by sensor size, stiffness, or micromotion.

- Collagen capsule thickness remained stable or increased somewhat over time for all sensor types.

- Collagen capsule formation was more prominent around the plastic sensor housing, whereas inflammatory cell density was greatest near the sensing electrode.

- Collagen density index (CDI) was relatively constant among tissue and sensor types. CDI was stable over time for all sensors except for the non-diabetic percutaneous Sof sensor.

**Inflammatory Cell Density:**
- Inflammatory cell density was greatest adjacent to the sensing electrode portion of the sensor.

- No significant differences in inflammatory cell density were observed between non-diabetic and diabetic tissue for any sensor type.

- Percutaneous Enlite sensors had significantly greater inflammatory cell density than percutaneous Sof sensors at 3 days.

- By 1 month however, no significant differences in inflammatory cell density were seen among sensors in the 0-50 µm zone.

- Inflammation for all sensor types increased or was constant over time, suggesting a chronic inflammatory response.

Microvessel Density:

- At 1 week, microvessel density was significantly greater adjacent to percutaneous Enlite sensors in non-diabetic tissue vs. diabetic, however no significant differences were observed between non-diabetic and diabetic tissue for any sensor type at 1 month.

- The non-diabetic percutaneous Enlite sensor had the greatest inflammatory cell density and microvessel density at early timepoints.

- Vascularity was generally stable over time for all sensor types in the 0-50 µm zone except for the diabetic percutaneous Sof sensor. In other zones, vascularity increased over time for all sensor types.
- At the 1-month timepoint, no significant differences in microvessel density were observed among sensors or in different tissue types.

Sensor performance:
- Sensor performance decreased considerably towards the 1-month timepoint.
  Sensor performance appeared worse for non-diabetic sensors judging from increased sensor failure rates during bolus events and over time.
- Sensitivity to glucose decreased steadily for all sensor types except for the non-diabetic Enlite sensor.
- The non-diabetic Enlite sensor had a greater sensitivity to glucose over time than the non-diabetic Sof sensor.
- Lag time was relatively similar among sensor types, tissue types, and over time.
- MARD values for diabetic sensors were considerably less than non-diabetic sensors for the Day 1 bolus, however all sensors had similar MARD values for the 1-week bolus.

13.4.7: Novelty and Impact

Specific Aim 4 employed the novel, outcome-based dosing regimen for inducing Type 1 diabetes in rats developed in Specific Aim 1. While the mode of inducing diabetes is not novel, as streptozotocin has commonly been used to induce diabetes in animal models, our variable dosing regimen is novel. Although many studies administer multiple doses of streptozotocin, these protocols are not outcome-based, which results in a percentage
of animals not becoming diabetic and being discarded from the study. Our method differs in that a variable number of streptozotocin doses are administered as needed, with 100% of our animals achieving the diabetic state.

This Specific Aim was also impactful in that it resulted in the development of an animal model capable of investigating the long-term tissue response to percutaneously-implanted functional Medtronic® glucose sensors. A novel animal protocol was developed, which involved the implantation of two different Medtronic® glucose sensors in rat dorsum. Most importantly, the highly effective Vetrap method for protecting and stabilizing the external sensor hubs and connected Medtronic® transmitters was developed, which was instrumental in preventing sensor extrusion over the 1-month study duration. Previous studies in our lab employed modified, commercial rat jackets to secure the external transmitters. These studies, which used only the Medtronic® Sof sensors, had a high rate of sensor extrusion for a shorter timepoint (3 weeks). Development of the Vetrap rat wrapping method reliably allowed for the investigation of the Medtronic® Sof and Enlite sensors for a period of 4 weeks. Additionally, the results of Specific Aim 4 have indicated no significant differences in most endpoints (collagen capsule thickness, collagen capsule density, inflammatory cell density, microvessel density) among sensor types at the 1-month timepoint, which may be indicative of the ability of the Vetrap wrapping method to sufficiently stabilize
external hardware and minimize the effects of micromotion. This animal protocol can thus be used in future studies to further investigate differences in glucose sensor design for short- and long-term durations.

13.4.8: Conclusion and Future Recommendations

Despite a consistent and reproducible diabetic model and an effective animal protocol that allowed for long-term evaluation of percutaneously-implanted, functional glucose sensors, few significant differences in endpoint parameters were observed in non-diabetic and diabetic tissue and among different sensor types by the 1-month timepoint. As previously mentioned, the novel Vetrap animal wrapping method may have sufficiently stabilized the external hardware and thus minimized or eliminated the effects of micromotion on the observed tissue responses. In addition, differences in sensor design may not have been pronounced enough to produce noticeable changes in tissue response over time.

One of the more interesting observations to note was that the diabetic foreign body response to indwelling sensors was not significantly different than the non-diabetic response. Though many studies have shown that inflammatory cell migration to the wound site is generally delayed in diabetic tissue, the results of this study showed equal numbers of inflammatory cells at all timepoints. Consistent with the literature however, the diabetic response to percutaneously-implanted glucose sensors was characterized by
prolonged and persistent inflammation. Interestingly, chronic inflammation was also observed adjacent to sensors in non-diabetic tissue. These results suggest that biocompatibility of electrochemical glucose sensors is an issue in non-diabetic and diabetic tissue and though these sensors are capable of producing raw signal for long durations, the surrounding tissue is adversely affected. For these sensors to be feasible for long-term patient care, modifications other than size, stiffness, and flexibility of the sensor must be considered. Indeed, the Enlite and Enlite 2 sensors, whose smaller, more flexible designs were intended to minimize implant micromotion and encourage a less aggressive tissue response, produced a greater inflammatory response than the older Sof sensor design. Previously observed in histological images, inflammatory cell density was highly concentrated in the region directly adjacent to the sensing electrode. This electrode, which represents a higher proportion of the Enlite and Enlite 2 sensors, is likely the reason why these sensors have a greater inflammatory cell density. Therefore, studies involving surface modification of these glucose sensors or application of therapeutic coatings may be applicable for improving long-term tissue response. In particular, a coating that can release nitric oxide without significantly affecting sensor signal could possibly reduce chronic inflammation and improve the surrounding tissue response. Because the diabetic tissue response is comparable to that in non-diabetic tissue for this particular model, it is possible that future studies involving this model can be primarily be conducted in non-diabetic rats. Previous glucose sensor studies in non-
diabetic rats still provide valuable information on tissue response and function and do not necessarily need to be redone in diabetic animals.

Throughout the study, no widespread trends were observed, however significant differences did appear occasionally at certain timepoints. Unfortunately, because of the number of variables being examined (percutaneously-implanted vs. fully-implanted, size, stiffness/flexibility, sensor composition/amount of tubing), the possibility exists that some of the significant outcomes were due to the familywise error rate, which is the probability of making one or more false discoveries (type 1 errors). Therefore, it may be necessary to utilize a correction method, such as the Bonferroni or Tukey correction, to determine the true level of significance. Additionally, we had hoped to perform correlation testing on the data to see if any of the measured histological endpoints affected either each other or the overall sensor performance. This correlation testing was complicated by the fact that the data were not normally distributed. In addition, distributions were not consistent among sets of data. Attempts to normalize the data via logarithm and square root transformations were thus unsuccessful. Determining the Spearman’s rank correlation coefficient was then considered, as it is a nonparametric measure of statistical dependence between two variables. However this method works ideally if the data are paired or if the sample sizes are equal among the groups being compared. Because of issues such as sensor extrusions, animal deaths, difficulty
sectioning/staining tissue samples, etc., sample sizes were rarely equal (for example, sample size of diabetic Enlite sensor inflammatory cell counts could be n=13 while sample size for microvessel counts was n=11). Because it would be inappropriate to edit data to make the sample sizes equal, we were unable to calculate Spearman’s rank correlation coefficient for our data sets. In addition, microvessel densities were not necessarily taken from the same animals as inflammatory cell densities due to sensor extrusion, etc. A future recommendation would be to ensure that all data are properly matched by animal so that equal sample sizes are present. The ability to perform correlation testing would have greatly strengthened the conclusions of this study and allowed us to better understand relationships among the measured parameters. Additionally, conducting the study to acquire paired data in the future would provide greater statistical strength.

Finally, future studies should focus on one sensor type before moving to multiple sensor designs. It is possible that the lack of consistent trends present in this study is the result of too many variables being examined at once. While all percutaneously-implanted glucose sensors were subjected to mechanical stress from external hardware, it was difficult to determine the level to which this micromotion contributed to the final tissue response as there were also differences in sensor design. Future studies should focus on isolating each of these variables as much as possible and testing each individually in a
single sensor type so that the true relationships and trends between the measured parameters can be determined.

The novel outcome-based dosing regimen for inducing Type-1 diabetes in rodents and the novel glucose sensor animal model developed in this study provide the ability to investigate the long-term tissue response to glucose sensors as well as sensor performance over time. Though significant trends were not observed in this study, this protocol contributes greatly to the field of glucose sensor research by laying the groundwork for future studies.

13.5: Challenges and solutions

Completion of specific aim 4 required much experimentation and trial and error in regards to 1) sensor implantation, 2) sensor and transmitter protection (rat wrapping methods), 3) prevention of sensor extrusion, and 4) determining correct dosages of glucose and insulin for challenges.

13.5.1: Sensor implantation

Although all Medtronic® sensors had introducer needles designed for sensor insertion through the skin, the thickness and looseness of rat skin proved problematic for these needles. When the introducer needles were inserted into the rat skin, the sensors would adhere to the skin instead of proceeding along the tract created by the needle. This often resulted in bending of the sensor, making subsequent implantation more difficult.
Various surgical techniques were then tested, including using 12-gauge needles as a trocar and bending of the introducer needle (for Enlite™ sensors). For the trocar method, the introducer needles were removed from the sensors. A 12-gauge needle was then used to create a “tunnel” through the subcutaneous space with an entry point and an exit point. The sensor, still attached to the plastic housing, was then inserted through one end of the 12-gauge needle. While pressing firmly on the plastic housing to ensure the sensor remained within the tissue, the 12-gauge needle was slowly withdrawn from the skin leaving the sensor in the subcutaneous space. The plastic housing was then attached to the skin with 3-0 nylon monofilament sutures. This technique, though promising in theory, often resulted in sensor extrusion due to formation of a gap between the wound site and the base of the sensor. One strategy for Enlite™ sensor implantation involved bending the introducer needle so that the sensor would be implanted parallel to the skin surface instead of directly into the tissue, as designed for human patients (Figure 105). A 12-gauge needle was used to make an entry point into the skin for the introducer needle. The modified introducer needle was then placed through the entry point and the spring-loaded mechanism was activated, releasing the sensor into the subcutaneous space. The bending of the introducer needle made this process extremely difficult, as the introducer needle did not retract effectively when bent.
Figure 105: Prior implantation method. A) Enlite\textsuperscript{TM} sensor with 90\degree introducer needle and implantation angle. B) Introducer needle was bent to allow sensor to be implanted within the subcutaneous tissue of the rat.

The final sensor implantation technique left the sensors and introducers intact. A 12-gauge needle was used to create an entry point for the introducer needles. The sensor was then inserted along with the introducer needle and the plastic housing was carefully adhered to the skin with super glue. The plastic housing was then sutured to the skin via the side tying platforms. The introducer needle was then removed. This strategy utilized the introducer needle, which was very useful in protecting the sensor until fully implanted, and prevented a significant gap between the sensor base and the insertion site. Sensor extrusion was greatly reduced upon development of this surgical procedure.

13.5.2: Sensor and transmitter protection (rat wrapping methods)

Once implanted, external sensor hubs and attached transmitters must be protected from the rat and the environment. Loose parts can get caught or pulled out, resulting in sensor extrusion. Originally, rat jackets were purchased from Lomir Biomedical, Inc ®.
These jackets were designed for specific weight ranges of rats (i.e. 150-250 g) however, and were often too loose or ill-fitting. Additionally, dermal inserts also purchased from Lomir were thin and did not cover sufficient portions of the rat’s back (Figure 106).

These jackets and dermal inserts were combined with ACE bandaging secured with Velcro strips. Unfortunately, the ACE bandaging, which was not waterproof, became loose over time due to moisture. Any loose fabric was easily accessible to the rats and quickly removed. The final solution involved wrapping the rats in Vetrap 3M animal wrap (St. Paul, MN), which is an elastic, waterproof material that adheres to itself. Wrapped properly, rats are unable to chew the material and expose external sensor parts.

Figure 106: Images of prior rat wrapping procedure. Rat jackets and dermal inserts (right) were purchased from Lomir Biomedical, Inc. ©. Lomir jackets and dermal inserts were paired with ACE bandaging to protect external hardware (left).
13.5.3: Prevention of sensor extrusion

Sensor extrusion was influenced by a number of factors: looseness of the rat skin/gap between wound site and sensor base, the tightness of sutures, and re-growth of rat hair. As mentioned previously, a significant gap between the sensor base and the insertion site can result in sensor extrusion. Over time, movement of the rat and the skin shifts the insertion site from the sensor base and the sensor is pulled out of the tissue. If sutures are too tight, they can sever through the rat skin and release the plastic housing, eventually resulting in sensor extrusion. Additionally, re-growth of rat hair can push the sensor hub upwards away from the skin and push the sensor out of the subcutaneous space. Modifications in surgical procedure addressed the rat skin gap. Additionally, the initial suture attaching the plastic housing to the skin was made snugly, but not tight. Subsequent knots in the suture were made tightly. Nair hair-removal cream was applied to the skin immediately prior to sensor implantation to remove and slow hair growth. The final solution involved super glue to attach the sensor hubs to the skin surface. Additionally, for the one-month time point, sutures were replaced as necessary.

13.5.4: Determining correct dosages for insulin/glucose challenges

Determining the correct dosages of solutions is essential in observing sensor performance. Dosages must be sufficient to be able to observe clear changes in blood glucose levels. Initially, only glucose boluses were administered, however even
moderate doses of glucose could raise blood glucose levels in diabetic rats to a level that was not within range of standard glucometers (>600 mg/dl). Administering the same dose to non-diabetic rats often did not result in noticeable changes in blood glucose levels. Eventually, rapid-acting insulin was administered to all rats so that an appropriate glucose load could be given afterwards. An overdose of insulin can lead to insulin shock however, so insulin doses were adjusted accordingly. Initial doses were obtained from a prior, unpublished study in our lab. These doses were administered to healthy rats that were under anesthesia (2% isoflurane in oxygen) for the duration of the experiment (~2-3 hours). Previous studies have indicated that anesthesia can have considerable effects on blood glucose levels. For this study, rats were placed under anesthesia only for the administration of the boluses in order to minimize possible anesthesia effects on blood glucose levels. Insulin and glucose dosages were then adjusted accordingly from the previous study.
Part VI: ONE-SIDED DORSAL WINDOW MODEL

Chapter 14: Examining the feasibility of using a one-sided dorsal window model to observe microvessel formation adjacent to nonfunctional subcutaneous glucose sensors in healthy and diabetic animals

14.1: Introduction

Window chamber models have often been used to visualize in situ cellular phenomena in living tissue\textsuperscript{180-182}. These chambers can be used for days to months in an animal, allowing for long-term serial investigations of microcirculation, wound healing, and cellular interactions\textsuperscript{183}. One type of window chamber, the two-sided dorsal skin-fold window chamber\textsuperscript{184-186} has been used in hamsters, mice, rats, and rabbits\textsuperscript{187-191} to monitor tumor growth\textsuperscript{188-189}, biomaterial interactions\textsuperscript{190, 192-193}, and tissue remodeling around implantable sensors\textsuperscript{183-184, 187}. The two transparent plates typically have a separation distance of 200 µm or less. This small distance provides an essentially two-dimensional view of the tissue, which may not be representative of the true tissue response. Another window chamber model, the one-side window adapted from the rodent mammary window of Shan\textsuperscript{189}, has been used to explore tissue remodeling around implantable sensors. The use of one window exposes the implant to a larger subcutaneous area and allows for a more realistic sensor-tissue interaction than that possible with a two-window model\textsuperscript{186, 188}. 
The use of window chambers for glucose sensor research was established by Gough\textsuperscript{183-184}, who had previously used dorsal skin fold window chambers to serially monitor changes in tissue surrounding implantable oxygen, glucose and/or temperature sensors in hamsters. These tissue changes were evaluated to determine their effect on sensors function. Sensor fluctuations were attributed to heterogeneous mass transfer in tissue from physiological changes in local microvascular perfusion, metabolite concentration, and variable oxygen distribution in tissue. Microvascular development was visualized using intravital microscopy.

For this pilot study, a one-sided window chamber model was used to directly image the vascularity surrounding non-functional subcutaneous glucose sensors in healthy and diabetic rats. These sensors were cut from the plastic housing and fully implanted beneath the dorsal one-sided window to observe changes in microvascular development adjacent to the sensor surfaces over 7 days.

**14.2: Materials and methods**

**14.2.1: Materials**

Nunc\textsuperscript{TM} Thermanox\textsuperscript{TM} coverslips (15 mm diameter, 0.2 mm thickness) were obtained from Electron Microscopy Sciences (Hatfield, PA). Sodium fluorescein was obtained from Sigma-Aldrich (St. Louis, MO).
14.2.2: *In vivo* studies

Four male Sprague-Dawley type (CD) rats (150-200g, Charles River Laboratories, Inc., Raleigh, North Carolina) were used for the percutaneous dorsal window chamber studies using nonfunctional sensors (n=8). Two rats were given streptozotocin to induce diabetes. All National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed. Approval for these studies was granted by the Duke University Institutional Animal Care and Use Committee prior to initiation of the studies. Four rats were used to demonstrate window implantation and microvessel characterization methods.

14.2.3: Sensor Preparation

The nonfunctional sensors were functional Sof™ and Enlite™ glucose sensors that had been removed from their plastic hub connector. This procedure was conducted using aseptic technique to minimize risk of infection.

14.2.4: Anesthesia

Rats were anaesthetized with 2.5% isoflurane (Baxter Healthcare Corp., Deerfield, IL) in oxygen at a flow of 1 l/min, which was adjusted to effect. Puralube® ocular lubricant (Pharmaderm, Melville, NY) was applied to each rat eye to prevent corneal drying. Rats were also kept on a water-based heating pad to prevent hypothermia during surgery.
14.2.5: Implantation of the one-sided dorsal window

Once rats were anesthetized, the dorsum was shaved and marking pen was used to place two 10mm diameter circles along the midline. The first circle was approximately 2 cm below the scapular region with the second circle approximately 5-6 cm below the first. The skin was cleaned with chlorhexidine (Baxter-Healthcare, Co. Deerfield, IL) and alcohol wipes in triplicate. The rat was then covered with a sterile drape, exposing only the area of interest. A 10-mm biopsy punch was used to create a 10-mm diameter circular full-thickness incision. After the skin was removed, blunt dissection was used to form a shallow pocket around the excised tissue to provide space for the clear coverslip (15mm diameter, 0.2mm thickness, with 10-12 holes inserted around the diameter for suturing) (Hatfield, PA).

One Sof™ and one Enlite™ sensor were prepared during surgery (in order to maintain sterility) by removing the sensor from the plastic housing. The non-functional Enlite™ sensor was positioned in the middle of the excised area nearest the scapulae. The non-functional Sof™ sensor was positioned in the middle of the second excised area nearer the lower back. Following sensor strip placement, windows were placed above the sensors and secured with 4-0 monofilament sutures (Figure 107). The wound was gently cleaned with hydrogen peroxide, followed by antibiotic ointment (Target Corporation, Minneapolis, MN).
Figure 107: Window chambers implanted in rat dorsum directly above nonfunctional, subcutaneous glucose sensor strips. Enlite sensor implanted cranial (right); Sof sensor implanted caudal (left).

Rats were monitored daily for signs of infection or inflammation and to ensure the window was still implanted. Additionally, windows were monitored to determine if sensors were still visible through the windows or if significant migration had occurred. Antibiotic ointment was applied for the first few days to the window area, and rats were housed singly and allowed access to food and water *ad libitum*.

14.2.6: Intravital microscopy

Microvessels around the sensors were imaged using a Zeiss Axioskop 2 Plus fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY), a 5.0x objective (Carl Zeiss, Inc., Thornwood, NY), a 75-W Fluor Arc xenon arc lamp (Osram GmbH, Augsburg, Germany), fluorescein filter set (excitation 450-490 nm, emission ≥ 515nm) (Figure 108). Images and videos were captured using a Q-imaging Retiga 1300r (QImaging, Inc.,
Surrey, BC) and Andor SOLIS software (South Windsor, CT).

Figure 108: Apparatus used for intravital microscopy of window chambers.

This optical system resulted in an empirically determined functional depth of field of approximately 100 µm. The rat was anesthetized with 2% isoflurane in oxygen in an induction chamber. The rat was then gently transported to the microscope stage and connected to a nose cone supplying isoflurane. Epi-illumination was used to focus the
sensor beneath the window. A 25-gauge butterfly syringe needle was then inserted into the tail vein. While under anaesthesia, a bolus of 10 mg of sodium fluorescein (Sigma-Aldrich, St Louis, MO) in 0.1 ml sterile saline was injected via the tail vein. A minimum of four locations along the sensor was captured immediately post fluorescein injection. When possible, images were also captured during injection to observe vessel patency as the fluorescein perfused the microvascular network beneath the window. Imaging during injection could only be accomplished for one sensor. The rat was imaged for approximately 10 minutes, which images captured for both the Sof™ and Enlite™ sensors.

14.2.7: Explantation

After 1 week, animals were anesthetized and the sensors were imaged once more. Surgical scissors were then used to excise the window with the surrounding and underlying tissue intact (the sensor was included with this tissue). The tissue samples were then flash frozen with liquid nitrogen and stored in a -80°C freezer for future use. Animals were then sacrificed with an intracardiac injection of 3M potassium chloride (KCl).

14.3: Results

Fully-implanted, nonfunctional sensors were placed underneath clear window chambers implanted in rat dorsum. Figure 109 illustrates intravital microscopy of subcutaneous
sensors in non-diabetic tissue. Microvessels are clearly visible adjacent to both Enlite and Sof sensors, though resolution is slightly cloudy in some regions.


Figure 110 illustrates intravital microscopy of subcutaneous sensors in diabetic tissue. Considerable cloudiness is observed even at the Day 4 timepoint, becoming worse by the Day 7 timepoint. No microvessels can be seen adjacent to the sensors through the optical windows.

14.4: Discussion and conclusion

This pilot study was conducted to evaluate the utility of a one-sided dorsal window model to image changes in microvascular architecture adjacent to two different non-functional sensors (Sof™ and Enlite™). While histology remains the standard option for characterizing the tissue response surrounding implanted sensors, its limitation is that it can only provide a static snap-shot of tissue composition and structure at the time of
explantation. So while differential and immunohistochemical stains allow identification of various cells and tissues, the ability to collect serial, real-time data from a single animal is not present.

Each rat was implanted with two window chambers, with a non-functional sensor placed beneath each. Rats were imaged on Days 4 and 7, and vascularization beneath the window was measured using intravital microscopy. The infusion of sodium fluorescein via tail vein injection permitted microcirculation characterization adjacent to the sensor surface. The microvessels appeared dark against the fluorescent tissue because hemoglobin strongly absorbs light between 525-580 nm^{194-195}.

Although the window chamber successfully provided a method for directly visualizing vessel formation in the tissue surrounding implanted sensors, some issues did arise during the course of the study. Window infection and tissue inflammation was always a concern, which was minimized by daily topical application of antibiotic ointment around the window perimeter. Clouding of the window, presumably protein build-up, was a considerable issue particularly in the diabetic rats. This clouding was visible in diabetic rat window chambers by Day 4 and became worse by Day 7. The film made focusing on the sensor and any surrounding microvessels problematic. Sensors themselves also appeared to become infected particularly in diabetic rats. Future studies involving window chambers and diabetic rats will need to address these issues. In
addition, exudate was often produced beneath the window chambers, which also prevented optimal focusing. Resolution was improved by aspirating the accumulated exudate with a syringe inserted several millimeters away from the window to prevent bleeding under the window\textsuperscript{196}. A 5.0x objective lens was the highest power used due to limited working distance between the window and the lens. The limited depth of field achieved using higher powers may have compromised image resolution. Variability in light intensity was considerable over the entire image, which prevented the use of a simple (automatic) threshold analysis to quantify vascularity. Finally, sensor migration beneath the window was substantial in some rats. This migration was most likely the result of animal movement. Sutures may be necessary in future studies to anchor and hold the subcutaneous sensor in place beneath the window. In most cases, gentle manipulation of the dorsum dermis permitted window repositioning over the migrated sensor. Typically, sensor migration occurred during the first 24 to 48 hours post implantation, after which tissue integration prevented further sensor migration. Cyclic sensor motion due to animal respiration was accounted for by capturing microscopic images at the end of expiration.

The utility of the one-sided dorsal window model to image changes in microvascularization adjacent to fully implanted, non-functional sensor strips was successfully demonstrated, however many issues must be addressed if the window is to
be used in longer-term (>7 days) studies.
Part VII: CONCLUSIONS AND FUTURE WORK

Chapter 15: Summary of work presented

15.1: Establishment of a diabetic animal model

Male CD (Sprague-Dawley type) rats weighing 150-200 g were given three consecutive daily doses of 40 mg/kg streptozotocin (STZ) on Days 1, 2, and 3. On Day 5, tail vein blood glucose was checked. If blood glucose was not within the target diabetic range of 350-600 mg/dl, rats received an additional dose of STZ. This procedure was repeated every 48 hours until all rats achieved target hyperglycemia. Non-diabetic rats were given similar doses of vehicle (saline/citrate), which had no effect on blood glucose. Stable hyperglycemia was maintained for the duration of the experiment (at least 21 days). There was a significant increase in the mean blood glucose for the STZ group when compared to the vehicle group for all days except day 0 when compared by a Student’s t-test (p < 0.0001*). Additionally, there was a significant difference in the mean body weight between the diabetic and non-diabetic groups for all days except day 0 when compared by a Student’s t-test (p < 0.0001*).

15.2: Infection response in healthy and diabetic animals

Control stainless steel compression plates and plates coated with a nitric-oxide releasing xerogel were attached to the femurs of 12 healthy adult rabbits. Both femurs were inoculated with 3x10⁶ CFU MSSA (methicillin-sensitive Staph aureus) for a period of 20 minutes before the surgical sites were sutured. After 7 days, the wound, device, and a
portion of bone were cultured. A muscle biopsy was removed and homogenized to quantify bacterial infection. Since the microbiologic data were not normally distributed, they were compared using an unpaired Wilcoxon Rank Sum Test. No significant difference between the control and NO-eluting devices was found, which may be a result of a variety of factors. The formulation of NO (20% AHAP3), while exhibiting the largest reduction in adherence of the bacterium *Pseudomonas* to glass, may not be adequate to combat a virulent strain of *Staph aureus*. In addition, this study differed greatly from previous studies in terms of animal model (rat to rabbit), implant material (silicone to stainless steel), implant location (subcutaneous to femur), and bacterial strain (mail-order MSSA to virulent MSSA osteomyelitis strain). The study did find a high correlation of temperature of the adjacent muscle at implantation with the ensuing bacterial content.

A novel dosing-regimen of streptozotocin based on a target blood glucose of 350-600 mg/dl was used to induce type-1 diabetes in adult male CD rats in this study. Stable hyperglycemia was maintained for at least 21 days. Two weeks after achieving the target hyperglycemia, control and diabetic rats received one stainless steel fracture plate and fixation screw on each anterior femur. One implant site per rat received an inoculum of $10^7$ CFU of methicillin-sensitive *Staphylococcus aureus*. After seven days, quantitative bacterial count was performed at explantation and no cross-over of bacteria
was detected from the inoculated side to the non-inoculated side. Infection after *S. aureus* inoculation in the presence of an implanted device was significantly higher in diabetic animals when compared to that of control animals (p = 0.0003) supporting the hypothesis that diabetes adversely affects the ability to fight infection in the presence of an indwelling device in an animal model.

**15.3: Nitric oxide as a means to mediate the tissue response to indwelling implants**

*In vivo* glucose recovery of subcutaneously implanted NO-releasing microdialysis probes was evaluated in a rat model using saturated NO solutions that provided a steady release of NO. A constant NO flux of 162 pmol cm$^{-2}$ s$^{-1}$ was perfused through the probe membrane for 8 hours daily. The *in vivo* effects of increased localized NO were evaluated by monitoring glucose recovery over a 14-day period. Beginning at 7 days, differences in glucose recovery between the control and NO-releasing probes were observed. At the 14-day time point, histological analysis revealed decreased inflammatory cell density at the probe surface and a thinner collagen capsule.

Polyurethane-coated wires with varying NO release properties were implanted subcutaneously in 17 Yorkshire piglets with time points of 3, 7, 21, and 42 days. Effects of NO release were analyzed using histological data. These data were analyzed using a non-parametric Wilcoxon rank sum test. Coatings with short NO release durations (i.e., 24 h) failed to reduce collagen capsule thickness at 3 and 6 weeks. Longer release
durations (3 and 14 d) however significantly reduced collagen capsule thickness at longer time-points. The acute inflammatory response was significantly effected by coatings with the longest duration and greatest dose of NO release. However these benefits were not realized at later time-points, suggesting that NO must be actively released in order to influence the inflammatory response.

15.4: Tissue response to functional glucose sensors in healthy and diabetic animals

The tissue response to percutaneously-implanted, functional glucose sensors was investigated in non-diabetic and diabetic tissue. A multi-dose regimen of streptozotocin was used to induce uncontrolled Type 1 diabetes in experimental rats. Three types of implantable glucose sensors, supplied by Medtronic® were used: Sof™ sensor, Enlite™ sensor, and Enlite 2™ sensor. Enlite™ and Enlite 2™ sensors represent newer, thinner and more flexible glucose sensor designs intended to promote a less aggressive foreign body response by reducing sensor micromotion. At 3 days, 1 week, and 4 weeks, tissue directly adjacent to the sensors was evaluated for collagen encapsulation, density of any collagen encapsulation, inflammatory response as measured via inflammatory cell density, and microvessel density. These endpoints were evaluated histologically via Masson’s trichrome, Hoechst, H&E, and CD31, and CD68 staining. Additionally, continuous functional sensor data was evaluated for sensor accuracy, attenuation, and lag time. All data were analyzed using the nonparametric Mann-Whitney U test.
Histological analyses revealed few significant differences in collagen thickness among different sensors, in different tissue types, or over time. In general, collagen capsule formation appeared to be balanced with inflammatory cell density. As inflammation increased adjacent to sensors over time, collagen capsule thickness decreased somewhat and stabilized. Collagen capsule formation was most evident adjacent to the plastic tubing portion of the sensor whereas inflammation was greatest adjacent to the sensing electrode. Likewise, few significant differences in collagen density index (CDI) were observed among sensor types, tissue types, or over time. CDI remained relatively constant over time for all sensors. Analysis of inflammatory cell density in general revealed a greater inflammatory response adjacent to percutaneous Enlite sensors, though these results were not significant. Additionally, inflammatory cell density was generally greater adjacent to non-diabetic sensors, however this result was also not significant. Inflammatory cell density increased or remained stable over time for all sensor types, suggesting that the presence of percutaneously-implanted sensors produces a chronic inflammatory response that does not resolve. Vascularity adjacent to implanted sensors remained generally stable over time, sometimes decreasing but not significantly. At the 1-month timepoint, no significant differences in vasculature were seen among sensor types. A balance also appears to exist for microvessel and inflammatory cell densities. The non-diabetic percutaneous Enlite sensor had the greatest microvessel density at earlier timepoints, while also having the greatest
inflammatory cell density. However, at later timepoints, microvessel density decreased somewhat as inflammation increased somewhat. Finally, analysis of sensor performance showed significant sensor failure at longer timepoints. Sensitivity decreased somewhat for all sensors except for the non-diabetic Enlite sensor, which in general had greater overall sensitivity in comparison to the non-diabetic Sof sensor. Lag time was relatively similar among all sensor types, tissue types, and over time. MARD values were considerably lower for diabetic sensors for the Day 1 bolus, but were generally similar for all sensors at the 1-week bolus.

15.5: Conclusions

The purpose of this dissertation was to investigate the tissue response to infectious burden and indwelling devices in both healthy and diabetic animal models. Using the drug streptozotocin, we developed a robust and reproducible model of uncontrolled Type 1 diabetes in rats. These diabetic rats had significantly higher HbA1c and a poorer immune response to infectious burden with and without the presence of an implant than non-diabetic animals. Tissue response to different types of implants were investigated; these included stainless steel fracture plates, stainless steel wires, and percutaneously-implanted wire electrode glucose sensors. Differences in glucose sensor design were analyzed in non-diabetic and diabetic rats. The response in different tissue types was not significantly different, and sensor design intended to reduce implant micromotion seemed to promote a greater inflammatory response. Nitric oxide therapy was also
investigated as a method to promote the wound healing response (as well as sensor performance in the case of glucose sensors) adjacent to implanted devices. Taken together, these results provide greater understanding on the wound healing responses adjacent to implanted devices in non-diabetic and diabetic animal models, which can be used in the development and optimization of indwelling devices and sensors.

Chapter 16: Recommendations for future work

This doctoral research aimed to understand the tissue response to implanted devices. These tissue responses were observed in healthy animal models, and then later in a diabetic animal model. We also explored the tissue response to infectious burden in healthy and diabetic animals. Finally, because nitric oxide has been shown to positively affect tissue response adjacent to implanted devices, we explored the tissue response to various implanted materials coated with this therapeutic agent. We observed the ability of nitric oxide to reduce collagen capsule thickness and reduce inflammation at implant interfaces. We also observed a poorer response to infection as well as a higher susceptibility to infection in diabetic animals. The diabetic animals exhibited prolonged inflammation and poor healing adjacent to implanted devices.

Areas requiring further investigation:
1. Could application of exogenous nitric oxide prove beneficial to the tissue response to implanted devices in diabetic animals?

2. Does micromotion have a noticeable effect on wound healing? What strategies could be developed to reduce micromotion, particularly in the case of percutaneously-implanted glucose sensors?

3. Quantification of the glucose gradient adjacent to implanted devices

4. Quantification of perfused microvessel density adjacent to implanted devices

16.1: Application of exogenous nitric oxide to indwelling implants in diabetic animal models

Diabetic wounds are characterized by nitric oxide deficiency. Previous studies have shown that delivery of NO to diabetic wound sites can partially enhance the wound healing response\(^{17}\). The results of the experiments of Specific Aim 3 have demonstrated many of the positive effects of nitric oxide on the tissue response to implanted devices. Originally, we had planned to apply these nitric oxide-releasing coatings to implants placed in diabetic animal models. However, a corporation was formed to commercialize the coatings, and this proprietary process was under the control of the new company. After discussion, it was clear that the new company was not comfortable collaborating with us and no further coatings would be provided by them. Nevertheless, exploration of NO efficacy in diabetic animals is a natural progression to the experiments presented in this dissertation. Non-commercial methods for delivering exogenous nitric oxide may be explored and prove to enhance the wound healing adjacent to implanted devices. In
addition, NO delivery may result in improved sensor performance, as indicated in
Specific Aim 3.

16.2: Coating strategies to reduce micromotion of percutaneously implanted glucose sensors

Mechanical irritation and micromotion are widely believed to be leading causes of increased inflammation, infection, and device failure\textsuperscript{197-199}. Percutaneous glucose sensors implanted into subcutaneous tissue often experience chronic micromotion from natural body movement and forces applied to external hardware. The direct and indirect effects of micromotion on device performance may be linked to premature device/implant failure due to increased fibrosis around the implant and heightened activity of inflammatory cells\textsuperscript{200-202}.

Proposed Study Design

Percutaneous implants likely undergo a greater deal of micromotion than subcutaneous implants. Various coatings can be explored that can be applied to the glucose sensors in order to minimize micromotion. One possibility consists of electrospun fibers that can be manufactured in various orientation and fiber diameters. These fibers could help anchor the sensor to the surrounding tissue and potentially minimize the foreign body response associated with micromotion. While it may not be possible to directly spin the fibers onto the sensor surface, the fibers can be used to form a sheet of fibers that can be folded and adhered to the sensor surface. Another possibility includes xerogel or
hydrogel coatings, which can encourage tissue ingrowth depending on their porosity. Finally, a solution as simple as securing biodegradable suture to the sensors could help minimize implant micromotion. These coatings would be analyzed in vitro to determine how securely they adhere to the implant. Once optimized, the coated glucose sensors will be placed percutaneously in diabetic and non-diabetic rats. Uncoated functional glucose sensors will also be implanted. External transmitters will be attached to the sensors as before. In addition, coated and uncoated nonfunctional glucose sensors will be fully implanted into the subcutaneous space for comparison. The percutaneous implants will be secured using Vetrap bandaging. After 1 week and 1 month timepoints, the wound sites, implants, and adjacent tissue will be explanted. Histological sections of the tissue and sensor will be quantified for inflammatory cell density, microvessel counts, and collagen deposition, to observe if a correlation exists between motion (due to sensor placement) and tissue response.

**16.3: Bioluminescence technique for the quantification of analyte gradients adjacent to implanted devices**

Adverse effects of the foreign body response such as collagen capsule formation and the infiltration of inflammatory cells can greatly disrupt the environment around an implanted device. In the case of glucose sensors, collagen capsule formation prevents the reliable diffusion of glucose to the sensor and results in inaccurate blood glucose measurements. In addition, inflammatory cells migrating to the sensor consume glucose and also contribute to an analyte gradient at the sensor interface. Quantifying the
glucose gradient around an implanted sensor can be useful in understanding the wound healing and tissue response.

A novel bioluminescence technique originally developed for tumor metabolite profiling can be used to quantify glucose gradients around implanted glucose sensors. The technique has been previously described\textsuperscript{203}. Briefly, the technique involves the rapid freezing of a tissue biopsy to halt metabolism and diffusion. Cryosections are then obtained from the biopsy, and analyte-specific dyes are then applied to the tissue sections. These dyes fluoresce in proportion to the concentration of analyte as the tissue thaws. Bioluminescence images are then digitally captured under a fluorescence microscope for analysis. The technique yields a snapshot of the metabolite distribution across a plane of tissue (Figure 111). Existing analyte profiling capabilities include glucose and lactate as well as markers for hypoxia, perfusion and other metabolism markers\textsuperscript{203-204}. 

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Figure 111: Glucose distribution map generated using a bioluminescence technique developed for metabolic mapping of tumors. This technique permits high-resolution visualization (20μm) of gradients of glucose and other metabolites in tissues.

16.4: Quantification of perfused microvessel density adjacent to implanted devices

While this doctoral research focused on microvessel density adjacent to implanted devices, a more complete analysis can be accomplished by quantifying the perfused microvessel density adjacent to these implants. By determining which microvessels are actually functional, it is possible to relate vascularity to glucose gradients around a device.
Hoechst 33342 is a nucleic acid stain that can be used to quantify perfused microvessel density. Immediately prior to animal sacrifice, Hoechst dye is injected via the tail vein and allowed to circulate within the bloodstream for 5 minutes. This allows the dye to adhere to functional microvessels. The animal is then sacrificed, and tissue surrounding implanted devices can be harvested, flash frozen with liquid nitrogen, and analyzed for perfused microvessel density.
Appendix A: NO Release Characteristics of Scaffolds

Table 16: Nitric oxide release from coatings doped at 18 mg/mL PROLI/NO as a function of polyurethane topcoat.

<table>
<thead>
<tr>
<th>NO release properties</th>
<th>Type of polyurethane topcoat</th>
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<tbody>
<tr>
<td></td>
<td>HP 93A</td>
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<tr>
<td>[NO]_{max} (pmol cm^{-2} s^{-1})</td>
<td>3128 ± 716</td>
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<tr>
<td>t_{max} (min)</td>
<td>8.4 ± 0.8</td>
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<tr>
<td>[NO]_{24h} (pmol cm^{-2} s^{-1})</td>
<td>5.45 ± 2.38</td>
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<tr>
<td>[NO]_{12h} (pmol cm^{-2} s^{-1})</td>
<td>0</td>
</tr>
<tr>
<td>[NO]_{24h} (pmol cm^{-2} s^{-1})</td>
<td>0</td>
</tr>
<tr>
<td>Total NO (µmol cm^{-2})</td>
<td>3.9 ± 0.4</td>
</tr>
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</table>

Table 17: Nitric oxide release from coatings doped at 18 mg/mL AEAP3 nanoparticles as a function of polyurethane topcoat.

<table>
<thead>
<tr>
<th>NO release properties</th>
<th>Type of polyurethane topcoat</th>
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<tbody>
<tr>
<td></td>
<td>HP 93A</td>
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<tr>
<td>[NO]_{max} (pmol cm^{-2} s^{-1})</td>
<td>108.8 ± 23.6</td>
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<tr>
<td>t_{max} (min)</td>
<td>9.2 ± 1.0</td>
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<tr>
<td>[NO]_{24h} (pmol cm^{-2} s^{-1})</td>
<td>28.04 ± 4.21</td>
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<tr>
<td>[NO]_{12h} (pmol cm^{-2} s^{-1})</td>
<td>10.94 ± 1.94</td>
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<tr>
<td>[NO]_{24h} (pmol cm^{-2} s^{-1})</td>
<td>1.02 ± 0.31</td>
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<tr>
<td>[NO]_{48h} (pmol cm^{-2} s^{-1})</td>
<td>0</td>
</tr>
<tr>
<td>Total NO (µmol cm^{-2})</td>
<td>2.7 ± 0.3</td>
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Table 18: Nitric oxide release from coatings doped at 36 mg/mL AEAP3 nanoparticles as a function of polyurethane topcoat.

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<th>NO release properties</th>
<th>HP 93A</th>
<th>HPU</th>
<th>HPU/TPU</th>
<th>TPU</th>
<th>TP-470</th>
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<tr>
<td>[NO]_{max} (pmol cm^{-2} s^{-1})</td>
<td>353.6 ± 87.9</td>
<td>324.2 ± 68.2</td>
<td>316.1 ± 71.3</td>
<td>189.2 ± 51.4</td>
<td>123.4 ± 36.1</td>
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<tr>
<td>t_{max} (min)</td>
<td>7.2 ± 0.9</td>
<td>6.6 ± 0.8</td>
<td>18.0 ± 2.8</td>
<td>72 ± 19</td>
<td>96 ± 21</td>
</tr>
<tr>
<td>[NO]_{6h} (pmol cm^{-2} s^{-1})</td>
<td>84.65 ± 10.61</td>
<td>92.95 ± 8.95</td>
<td>111.11 ± 13.51</td>
<td>68.18 ± 7.12</td>
<td>86.79 ± 6.94</td>
</tr>
<tr>
<td>[NO]_{12h} (pmol cm^{-2} s^{-1})</td>
<td>30.28 ± 4.12</td>
<td>35.88 ± 3.32</td>
<td>38.93 ± 3.16</td>
<td>41.56 ± 3.56</td>
<td>51.54 ± 5.84</td>
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<td>[NO]_{24h} (pmol cm^{-2} s^{-1})</td>
<td>2.80 ± 0.71</td>
<td>3.64 ± 0.42</td>
<td>5.12 ± 1.02</td>
<td>10.05 ± 1.80</td>
<td>10.91 ± 1.11</td>
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<tr>
<td>[NO]_{48h} (pmol cm^{-2} s^{-1})</td>
<td>0.96 ± 0.17</td>
<td>1.12 ± 0.06</td>
<td>1.35 ± 0.31</td>
<td>2.58 ± 0.64</td>
<td>2.84 ± 0.43</td>
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<tr>
<td>[NO]_{72h} (pmol cm^{-2} s^{-1})</td>
<td>0</td>
<td>0.92 ± 0.04</td>
<td>1.15 ± 0.16</td>
<td>1.28 ± 0.08</td>
<td>1.56 ± 0.17</td>
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<tr>
<td>Total NO (μmol cm^{-2})</td>
<td>6.1 ± 0.6</td>
<td>6.1 ± 0.5</td>
<td>6.0 ± 0.5</td>
<td>5.9 ± 0.6</td>
<td>5.8 ± 0.5</td>
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</table>
Table 19: Nitric oxide release from coatings doped at 18 mg/mL or 36 mg/mL MPTMS nanoparticles with a HPU/TPU polyurethane topcoat.

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Appendix B: Processed Data Files (Medtronic, Inc.)

Example of processed data file provided by Medtronic, Inc. File shows time of each raw sensor signal, raw signal, computer sensor glucose values, blood glucose values, etc.

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Appendix C: Sensor Function Information (Proprietary)

Each Medtronic® sensor has a proprietary algorithm used to compute sensor glucose (SG) values from raw response current values. These algorithms have a logic to track for abrupt changes in the raw sensor response current. These abrupt changes are filtered out, which can be observed in some of the Bolus Event plots.

Conditions that could trigger the filtering:

1. The raw sensor response current is out of range (under 6 nA or over 200 nA).

2. An artifact, which is caused by a rapid non-physiological drop in raw sensor response current, is detected. This occurrence can last 90 minutes.

3. Very rapid changes in raw sensor response current or large amounts of noise.
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