Improving Indwelling Glucose Sensor Performance: Porous, Dexamethasone-Releasing Coatings that Modulate the Foreign Body Response

by

Suzana Gabriela Vallejo-Heligon

Department of Biomedical Engineering
Duke University

Date: __________________________
Approved:

___________________________
William “Monty” Reichert, Co-Supervisor

___________________________
Bruce Klitzman, Co-Supervisor

___________________________
Natalie A. Wisniewski

___________________________
Fan Yuan

___________________________
Howard Levinson

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

Improving Indwelling Glucose Sensor Performance: Porous Dexamethasone-Releasing Coatings that Modulate the Foreign Body Response

by

Suzana Gabriela Vallejo-Heligon

Department of Biomedical Engineering
Duke University

Date:_______________________

Approved:

___________________________

William “Monty” Reichert, Co-Supervisor

___________________________

Bruce Klitzman, Co-Supervisor

___________________________

Natalie A. Wisniewski

___________________________

Fan Yuan

___________________________

Howard Levinson

An abstract of a 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

Inflammation and the formation of an avascular fibrous capsule have been identified as the key factors controlling the wound healing associated failure of implantable glucose sensors. Our aim is to guide advantageous tissue remodeling around implanted sensor leads by the temporal release of dexamethasone (Dex), a potent anti-inflammatory agent, in combination with the presentation of a stable textured surface.

First, Dex-releasing polyurethane porous coatings of controlled pore size and thickness were fabricated using salt-leaching/gas-foaming technique. Porosity, pore size, thickness, drug release kinetics, drug loading amount, and drug bioactivity were evaluated. In vitro sensor functionality test were performed to determine if Dex-releasing porous coatings interfered with sensor performance (increased signal attenuation and/or response times) compared to bare sensors. Drug release from coatings monitored over two weeks presented an initial fast release followed by a slower release. Total release from coatings was highly dependent on initial drug loading amount. Functional in vitro testing of glucose sensors deployed with porous coatings against glucose standards demonstrated that highly porous coatings minimally affected signal strength and response rate. Bioactivity of the released drug was determined by monitoring Dex-mediated, dose-dependent apoptosis of human peripheral blood derived monocytes in culture.
The tissue modifying effects of Dex-releasing porous coatings were accessed by fully implanting Tygon® tubing in the subcutaneous space of healthy and diabetic rats. Based on encouraging results from these studies, we deployed Dex-releasing porous coatings from the tips of functional sensors in both diabetic and healthy rats. We evaluated if the tissue modifying effects translated into accurate, maintainable and reliable sensor signals in the long-term. Sensor functionality was accessed by continuously monitoring glucose levels and performing acute glucose challenges at specified time points.

Sensors treated with porous Dex-releasing coatings showed diminished inflammation and enhanced vascularization of the tissue surrounding the implants in healthy rats. Functional sensors with Dex-releasing porous coatings showed enhanced sensor sensitivity over a 21-day period when compared to controls. Enhanced sensor sensitivity was accompanied with an increase in sensor signal lag and MARD score. These results indicated that Dex-loaded porous coatings were able to elicit a favorable tissue response, and that such tissue microenvironment could be conducive towards extending the performance window of glucose sensors in vivo.

The diabetic pilot animal study showed differences in wound healing patterns between healthy and diabetic subjects. Diabetic rats showed lower levels of inflammation and vascularization of the tissue surrounding implants when compared to their healthy counterparts. Also, functional sensors treated with Dex-releasing porous coatings did not show enhanced sensor sensitivity over a 21-day period. Moreover, increased in sensor
signal lag and MARD scores were present in porous coated sensors regardless of Dex-loading when compared to bare implants. These results suggest that the altered wound healing patterns presented in diabetic tissues may lead to premature sensor failure when compared to sensors implanted in healthy rats.
Dedication

I would like to dedicate this work to my mother, Suzana and my aunt, Olga. Thank you for always being there for me!

Quiero dedicarle este trabajo a mi mamá, Suzana y mi tía, Olga. ¡Gracias por siempre estar presente!
Contents

Abstract ........................................................................................................................................iv

Dedication .........................................................................................................................................vii

List of Tables .....................................................................................................................................xv

List of Figures ..................................................................................................................................xvi

Acknowledgements ......................................................................................................................xxii

1. Chapter 1: Introduction ................................................................................................................1
   1.1. Clinical Significance .................................................................................................................1
   1.2. Hypothesis .................................................................................................................................2
   1.3. Approach .................................................................................................................................2

2. Chapter 2: Specific Aims ................................................................................................................5
   2.1. Specific Aim 1: Fabrication and characterization of porous, dexamethasone-
       releasing coatings for implantable glucose sensors .................................................................5
   2.2. Specific Aim 2: Characterization of the foreign body response to porous,
       dexamethasone-releasing coatings in healthy and diabetic animal models .......................6
   2.3. Specific Aim 3: Evaluation of the effects of porous, dexamethasone-releasing
       coatings on percutaneous SOF-Sensor Medtronic MiniMed™ glucose sensors in
       healthy and diabetic rats ............................................................................................................7

3. Chapter 3: Background ................................................................................................................10
   3.1. Clinical Need ............................................................................................................................10
   3.2. Tissue Response to Implantable Devices .................................................................................11
   3.3. In vivo Failure of Implantable Glucose Sensors and Glucose Transport
       Implications .................................................................................................................................12
3.4. Biomaterial Strategies to Reduce Inflammation .................................................. 17
3.5. Tissue Response to Textured Surfaces ................................................................. 20
3.6. Strategies to Improve In Vivo Glucose Sensor Biocompatibility ...................... 22
3.7. Medtronic MiniMed SOF-SENSOR™ Glucose Sensors .................................. 24
3.8. Diabetic Wound Healing ....................................................................................... 27
3.9. Diabetic Animal Models ....................................................................................... 30
3.9.1 Spontaneous Models of Type I Diabetes .......................................................... 31
3.9.2 Chemically Induced Models of Type I Diabetes ............................................... 32
4. Chapter 4: Specific Aim 1 - Fabrication and characterization of porous, dexamethasone-releasing coatings for implantable glucose sensors ......................... 34
   4.1. Synopsis: ............................................................................................................. 34
   4.2. Introduction: ....................................................................................................... 35
   4.3. Materials and Methods: ..................................................................................... 39
      4.3.1. Fabrication of Dex-Releasing Porous Coatings ........................................ 39
      4.3.2. Scanning Electron Microscopy (SEM) ...................................................... 44
      4.3.3. Micro-Computed Tomography (Micro-CT)) ............................................ 44
      4.3.4. Differential Scanning Calorimetry (DSC) .................................................. 45
      4.3.5. High Performance Liquid Chromatography (HPLC) ............................ 46
      4.3.6. Dexamethasone Release Studies ............................................................... 46
      4.3.7. Dexamethasone Loading Efficiency ......................................................... 47
      4.3.8. Sensor Response ......................................................................................... 47
      4.3.9. Peripheral Blood Derived Human Monocyte Isolation ............................. 48
4.3.10. Dex-treated Media Preparations for Apoptosis Assays.................................49
4.3.11. Annexin-V Apoptosis Assays......................................................................50
4.3.12. Statistical Analysis.......................................................................................51
4.4. Results:................................................................................................................51
4.4.1. Coating Pore Size, Thickness and Porosity.......................................................51
4.4.2. Differential Scanning Calorimetry.....................................................................54
4.4.3. Dexamethasone Loading and Retention after Gas-Foaming.............................54
4.4.4. Dexamethasone Release ..................................................................................57
4.4.5. Sensor Response Time and Signal Attenuation .................................................60
4.4.6. Bioactivity of Dex-Releasing Porous Coatings..................................................63
4.5 Discussion:.............................................................................................................65
4.6 Conclusions:............................................................................................................71

5. Chapter 5: Specific Aim 2 - Characterization of the foreign body response to porous, dexamethasone-releasing coatings in healthy and diabetic animal models..............73

5.1. Synopsis:...............................................................................................................73
5.2. Introduction:..........................................................................................................74
5.3. Materials and Methods:.......................................................................................76
5.3.1. Fabrication of Dex-Releasing Porous Coatings...............................................76
5.3.2. Preparation of Implants....................................................................................77
5.3.3. Implantation Procedure....................................................................................78
5.3.4. Dexamethasone Systemic Effects Study in Healthy Rats.................................81
5.3.5. In-vivo Evaluation of Dexamethasone-Releasing Coatings in Healthy Rats...81
5.3.6. Generation, Monitoring and Implantation of Diabetic Rat Animal Model .... 82
5.3.7. Evaluation Histological Samples................................................................. 83
5.3.8. Hematoxylin & Eosin (H&E) Staining......................................................... 83
5.3.9. Masson’s Trichrome Staining ................................................................. 84
5.3.10. CD31 Immunochemical Staining ............................................................ 85
5.3.11. CD68 Immunochemical Staining ............................................................ 86
5.3.12. Histological Image Acquisition and Analysis ......................................... 87
5.3.13. Collagen Capsule Density ...................................................................... 88
5.3.14. Microvessel and Macrophage Density .................................................... 88
5.3.15. Statistical Analysis .................................................................................. 89
5.4. Results: ........................................................................................................... 91
5.4.1. Dexamethasone Systemic Effects Optimization in healthy rats ............. 91
5.4.2. H&E Evaluation of Subcutaneous Tygon® Tubing Implants in Healthy Rats 91
5.4.3. CD31 Evaluation of Subcutaneous Tygon® Tubing Implants in Healthy Rats ................................................................................................. 94
5.4.4. Collagen Density of Tissue Surrounding Tygon® Tubing Implants in Healthy Rats ................................................................................................. 96
5.4.5. Macrophage and Vessel Density of Tissue Surrounding Tygon® Tubing Implants in Healthy Rats ................................................................................................. 99
5.4.6. H&E Evaluation of Subcutaneous Tygon® Tubing Implants in Diabetic Rats ................................................................................................. 102
5.4.7. CD31 Evaluation of Subcutaneous Tygon® Tubing Implants in Diabetic Rats. ................................................................................................. 104
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4.8. Collagen Density of Tissue Surrounding Tygon® Tubing Implants in Diabetic Rats</td>
<td>106</td>
</tr>
<tr>
<td>5.4.9. Macrophage and Vessel Density of Tissue Surrounding Tygon® Tubing Implants in Diabetic Rats</td>
<td>109</td>
</tr>
<tr>
<td>5.5. Discussion:</td>
<td>112</td>
</tr>
<tr>
<td>5.6. Conclusions:</td>
<td>119</td>
</tr>
<tr>
<td>6.1. Synopsis:</td>
<td>121</td>
</tr>
<tr>
<td>6.2. Introduction:</td>
<td>123</td>
</tr>
<tr>
<td>6.3. Materials and Methods:</td>
<td>126</td>
</tr>
<tr>
<td>6.3.1. Fabrication of Dex-Releasing Porous Coatings</td>
<td>126</td>
</tr>
<tr>
<td>6.3.2. Preparation of Functional Sensor Implants</td>
<td>126</td>
</tr>
<tr>
<td>6.3.3. Implantation of Functional Glucose Sensors</td>
<td>128</td>
</tr>
<tr>
<td>6.3.4. In-vivo Evaluation of Functional Glucose Sensors in Healthy Rats</td>
<td>132</td>
</tr>
<tr>
<td>6.3.5. Generation, Monitoring and Implantation of Functional Glucose Sensors in Diabetic Rats</td>
<td>132</td>
</tr>
<tr>
<td>6.3.6. Continuous Glucose Monitoring</td>
<td>133</td>
</tr>
<tr>
<td>6.3.7. Glucose Sampling</td>
<td>134</td>
</tr>
<tr>
<td>6.3.8. Glucose Bolus Tests</td>
<td>134</td>
</tr>
<tr>
<td>6.3.9. Explantation Procedure</td>
<td>135</td>
</tr>
<tr>
<td>6.3.10. Evaluation Histological Samples</td>
<td>135</td>
</tr>
<tr>
<td>6.3.11. Hematoxylin &amp; Eosin (H&amp;E) Staining</td>
<td>136</td>
</tr>
</tbody>
</table>
6.3.12. Masson’s Trichrome Staining ................................................................. 136
6.3.13. CD31 Immunochemical Staining .......................................................... 137
6.3.14. CD68 Immunochemical Staining ......................................................... 138
6.3.15. Histological Image Acquisition and Analysis ....................................... 140
6.3.16. Collagen Capsule Density ................................................................. 140
6.3.17. Microvessel and Macrophage Density ................................................. 141
6.3.18. Sensitivity Measurements .................................................................. 141
6.3.19. Mean Average Relative Difference (MARD) ....................................... 142
6.3.20. Sensor Lag Time ................................................................................. 142
6.3.21. Statistical Analysis ............................................................................ 143
6.4. Results: ................................................................................................. 143
   6.4.1. H&E and Macrophage Analysis Healthy Rats ....................................... 143
   6.4.2. Collagen and Vessel Density Analysis Healthy Rats ........................... 148
   6.4.3. Continuous Glucose Monitoring and Glucose Bolus Tests in Healthy Rats .. 154
   6.4.4. H&E and Inflammatory Cell Analysis Diabetic Rats ........................... 159
   6.4.5. Collagen and Vessel Density Analysis Diabetic Rats ........................... 164
   6.4.6. Continuous Glucose Monitoring and Glucose Bolus Tests in Diabetic Rats .169
6.5 Discussion: ............................................................................................ 174
6.6 Conclusions: ......................................................................................... 180

7. Chapter 7: Overview, Conclusions and Future Work .................................. 183
   7.1. Summary of Work .............................................................................. 183
7.1.1. Porous, Dexamethasone-Releasing Coating Development and Characterization ................................................................. 183

7.1.2. Tissue Response to Fully Subcutaneous Porous, Dexamethasone-Releasing Coatings in Healthy and Diabetic Tissues ................................................................. 183

7.1.3. Tissue Response to Porous, Dexamethasone-Releasing Coatings and Subsequent Effects on Glucose Sensor Function in Healthy and Diabetic Rats ..... 184

7.2. Conclusions: .................................................................................................................................................. 185

7.3. Future Work: ................................................................................................................................................. 186

7.3.1. Determine the Role of Macrophage Phenotype on Sensor Performance ...... 186

7.3.2. Role of Mechanical Irritation on Sensor Response ........................................ 188

7.3.3. Novel Fully Implantable Sensor Technology .................................................. 189

7.3.4. Immunomodulatory Coatings ............................................................................ 190

References .................................................................................................................................................... 194

Biography ..................................................................................................................................................... 208

Publications: ................................................................................................................................................ 209

Oral Presentations: ..................................................................................................................................... 209

Poster Presentations: ................................................................................................................................ 210

Awards:....................................................................................................................................................... 211
List of Tables

Table 1. Strategies to Mediate the Tissue Response to Glucose Sensor Implantation......23

Table 2. Porogen and Dex added to stock polymer solution to produce coatings different porosities and Dex loadings .................................................................39

Table 3. Physical Characteristics of Porous Dex-releasing Coatings (n=6) .................54

Table 4. Loading efficiency and release summary of Dex incorporated into porous polyurethane coatings by salt-leaching/gas-foaming method. (n=10) .........................56

Table 5. In vitro response of sensors deployed with Dex-releasing polyurethane porous coatings. (n=6) ..................................................................................62

Table 6. Porogen and Dex added to stock polymer solution to produce coatings different porosities and Dex loadings ........................................................................76

Table 7. Collagen density in tissue adjacent to Tygon® implants in healthy rats (n=7) ...96

Table 8. Collagen density in tissue adjacent to Tygon® implants in diabetic rats (n=7) 106

Table 9. Collagen density in tissue adjacent to functional sensor implants in healthy rats (n=5 or greater). ..................................................................................148

Table 10. Sensor Lag during glucose bolus tests for healthy rats (n=5 or greater). .......158

Table 11. Mean Average Relative Deviation during glucose bolus test for healthy rats (n=5 or greater). .................................................................159

Table 12. Collagen density in tissue adjacent to functional sensor implants in diabetic rats (n=3) ..................................................................................164

Table 13. Sensor Lag during glucose bolus tests for diabetic rats (n=3) ....................170

Table 14. Mean Average Relative Deviation during glucose bolus test for diabetic rats (n=3). ...............................................................................................171
List of Figures

Figure 1. Schematic showing the effects of the foreign body response on sensors and the proposed Dex-releasing porous coating system......................................................4

Figure 2. Time Course of the Foreign Body Response to Implantable Device [19]. ..........12

Figure 3. Foreign Body Response leads to premature Glucose Sensor Failure...............16

Figure 4. Schematic of the Medtronic MiniLink™ System. The system is composed of a sensor that measures glucose levels via an electrochemical reactions. The current signal produced at the sensor tip can be transmitted wirelessly via an external transmitter to a computer or insulin pump [81]..................................................................................................................26

Figure 5. Fabrication of Dexamethasone-Releasing Porous Coatings..........................40

Figure 6. Gas-Foaming/ Salt-leaching produces was employed to generate an interconnected pore network in dexamethasone-releasing porous coatings.........................41

Figure 7. Pictures and ESEM images of porous coatings created via salt leaching/gas-foaming technique. (A) Picture of Medtronic MiniMed Sof-Sensor™ with and without highly porous Dex-releasing coatings. (B) ESEM of exterior surface and (C) cross-section of 90 % Porosity – 2.9 wt % Dex coatings. Arrows indicate pore diameters (B) and coating thickness (C). (n=6)..........................................................................................................................43

Figure 8. Representative micro-CT images of porous coatings created via salt leaching/gas-foaming technique with decreasing porogen fraction. Coatings of different morphologies were created by varying the ammonium bicarbonate porogen concentrations of (A-B) 90%, (C) 60%, and (D) 30% are shown. Addition of Dex did not disrupt scaffold structure of (A) 0 wt % Dex and (B-D) 2.9 wt %. Corresponding 3D volume renderings of porous structures are also shown, individual colors represent interconnected void regions of coatings (E-H). (n=6) ..................................................................................53

Figure 9. Differential Scanning Thermographs of dexamethasone loaded porous coatings. Dex is found in an amorphous state within the scaffolds. (A) Initial loading amounts of Dex were increased from 0.7-2.9 wt % Dex. Dex is found in a similar crystal states in scaffolds with high (2.9 wt %) and low (0.7 wt %) initial loading amounts. Actual loading amounts are listed in Table 3. (B) Changes in coating porosity did not affect crystal state of Dex within scaffolds. (n=6) ..................................................................................55
Figure 10. Cumulative release of dexamethasone from porous coatings. (A) Initial loading amounts of dexamethasone (0.7, 1.4 and 2.9 wt %) and (B) original porogen content (90, 60 and 30% Porosity) were varied. Dexamethasone release from coatings shows high dependency on initial loading. * denotes p<0.05 or less compared to highlighted treatments (n=10).

Figure 11. Response of sensors with scaffolds of (A) varying porosities and (B) varying Dex loading concentrations compared to the response of bare sensors (without coatings) and sensors with non-porous coatings. Sensors were subjected to a glucose challenge by exposing them to a series of glucose concentrations in the following order: 0 mg/dL, 100 mg/dL, 200 mg/dL, 400 mg/dL and 0 mg/dL in PBS at 45 min intervals at 37 °C in stirred conditions. Decreasing coatings porosity resulted in increased in sensor response time and attenuation, while changes in Dex-loading did not affect sensor signal when compared to bare sensors. Data presented are representative traces of 6 independent tests. (n=6).

Figure 12. Dose Response of Human Peripheral Blood Derived Monocytes to Dex released from porous coatings. Monocytes were incubated in medium treated with porous coatings ranging from 0.7-2.9 wt % in initial Dex loading. Dex released from scaffolds induced apoptosis of monocytes in a dose and time dependent manner. There was a strong dose dependency on monocyte apoptosis and initial Dex coating loading after 48 and 72 hrs of incubation. * denotes P < 0.05 or less compared to untreated medium (n = 8).

Figure 13. Picture of Tygon® tubing implants with and without porous, Dex-releasing coatings.

Figure 14. Implantation procedure of Tygon® implants that were either bare, coated with Dex-free porous coatings or Dex-releasing porous coatings. Black box highlights 12 gauge needle and implant. Red boxes highlight the minimal cutaneous wound and the implant location within the subcutaneous space upon explantation.

Figure 15. Porous dexamethasone-releasing coatings were implanted in the dorsum of rats. Configuration of subcutaneous implants in the dorsum of rats. Implants were randomly rotated through the positions shown above.

Figure 16. Photomicrographs of implants coated with Dex-loaded porous coatings after 21 days in rats. a) Masson’s trichrome images were used to compute the collagen density of the foreign body capsule. Four random 50x50 µm² fields (red squares) were analyzed per image. CD68 (b) and CD31 (c) images were used to calculate the density of...
macrophages and microvessels respectively around implants. For each image, a “ring-like” 100 μm wide zone beginning from the edge of the implant (white dotter line) and moving outward (red dotted line) was defined and analyzed. Scale bars = 200 μm for (a) and 400 μm for (b) & (c).

Figure 17. H&E stained images of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in Healthy Rats. Bare and Dex-free implants show high levels of inflammation at days 3, 7. Tygon® implants deployed with Dex-releasing porous coatings show decreased inflammation at days 3 and 7. By day 21, a thin ring of cells was visible on the surface of Dex-releasing porous coatings. Error bars = 200 µm.

Figure 18. CD31 photomicrographs of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in Healthy Rats. No difference in vascularity between implants was observed for days 3 and 7. Dex-free porous coated Tygon® implants showed increased vascularity by day 14. Both Dex-free and Dex-Releasing porous coated Tygon® implants showed increased vascularity by Day 21. Error bars = 200 µm.

Figure 19. Masson’s Trichrome images of Tygon® implants and subcutaneous tissue after 3, 7, 14, and 21 days in Healthy Rats. By day 21, the formation of organized collagen capsule was observed on bare sensor implants. Porous implants regardless of Dex-loading were able to disrupt the formation of this capsule. Error bars = 200 µm.

Figure 20. Density of macrophages within the 100 μm zone surrounding Tygon® implants in Healthy Rats. The tissues surrounding Dex-free implants were significantly inflamed when compared to the tissue surrounding the implants with Dex releasing coatings at days 3, 7 and 14. However, by day 21 there was no difference in the number of inflammatory cells surrounding the implants.

Figure 21. Density blood vessels within the 100 μm zone surrounding Tygon® implants in Healthy Rats. The tissues surrounding implants showed no differences in vascularity at days 3 and 7. On day 14, vascularity around Dex-free porous implants significantly increased. By day 21, vascularity for all porous implants regardless of Dex-loading had significantly increased when compared to controls.

Figure 22. H&E stained images of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in diabetic rats. Error bars = 200 µm.

Figure 23. CD31 photomicrographs of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in diabetic rats. Error bars = 200 µm.
Figure 24. Masson’s trichrome images of Tygon® implants and subcutaneous tissue after 3, 7, 14, and 21 days in diabetic rats. Error bars = 200 µm.

Figure 25. Density of macrophages within the 100 µm zone surrounding Tygon® implants in Diabetic Rats. Macrophage density increased with time for all implants regardless of treatment. Tissue surrounding Dex-releasing coatings had the lowest macrophage densities for all time points. Macrophage densities were on average lower in diabetic tissues when compared to healthy rats.

Figure 26. Density blood vessels within the 100 µm zone surrounding Tygon® implants in Diabetic Rats. The tissues surrounding implants showed no differences in vascularity at any time points. On day 14 and 21, vascularity around Dex-free porous implants was lower when compared to healthy rat tissues. By day 21, vascularity for all porous implants regardless of Dex-loading was lower when compared to healthy rat controls.

Figure 27. Picture of Medtronic MiniMed glucose Sensor™ with and without highly porous Dex-releasing coatings.

Figure 28. Configuration of percutaneous Medtronic MiniMed SOF-SENSOR™ Sensor in the dorsum of rats. Implants were randomly rotated through the positions shown above.

Figure 29. Implantation Procedure of Functional Medtronic MiniMed SOF-SENSOR™ Sensors.

Figure 30. H&E stained images of Functional Sensor Implants and subcutaneous tissue after 3, 7 and 21 days in Rats. Bare and Dex-free implants show high levels of inflammation at days 3, 7 and 21. Sensors deployed with Dex-releasing porous coatings show decreased inflammation at days 3 and 7. By day 21, a thin ring of cells was visible on the surface of Dex-releasing porous coatings. Scale bars = 200 µm.

Figure 31. CD68 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in Rats. High levels of CD68 positive cells were observed surrounding Dex-free percutaneous implants. Sensors deployed with Dex-releasing porous coatings had low levels of CD68 positive cells. Scale bars = 500 µm.

Figure 32. Density of Macrophages within the 100 µm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in Rats. The tissues surrounding Dex-
releasing porous coatings has significantly less macrophages than tissues surrounding Dex-free porous and Bare Sensor implants at days 3 and 7.

Figure 33. Masson’s trichrome images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in healthy rats. By day 21, the formation of organized collagen capsule was observed on bare sensor implants. Porous implants regardless of Dex-loading were able to disrupt the formation of this capsule. Scale bars = 200.

Figure 34. CD31 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in healthy rats. No difference in vascularity between implants was observed for days 3 and 7. Dex-free Porous coated and Dex-Releasing porous coated sensors showed increased vascularity by Day 21. Scale bars = 500 µm.

Figure 35. Density of blood vessels within the 100 µm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in healthy rats. The tissues surrounding implants showed no differences in vascularity at days 3 and 7. By day 21, vascularity for all porous implants regardless of Dex-loading had significantly increased when compared to controls.

Figure 36. Plot of sensor sensitivities of implanted Medtronic MiniMed SOF-Sensor glucose sensors. Dex-releasing porous coated sensors retained high sensitivity levels through the 21 day period when compared to Dex-free porous coated and bare controls. Sensitivity of Dex-free porous and bare controls decreased after day 12.

Figure 37. Representative Glucose Bolus Tests of implanted Medtronic MiniMed SOF-Sensor glucose sensors at (a) 1 day, (b) 3 days, (c) 7 days, (d) 14 days, and (f) 21 days in healthy rats. Bare and Dex-free porous coated controls began to fail by day 14 post-implantation. Dex-Releasing porous coated sensors responded to glucose challenges through day 21 post-implantation. All coated sensors regardless of Dex-loading had increased sensor lag when compared to controls.

Figure 38. H&E stained images of Functional Sensor Implants and subcutaneous tissue after 3, 7 and 21 days in diabetic rats. Scale bars = 200 µm.

Figure 39. CD68 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in diabetic rats. Little to no macrophages were observed in the surface of percutaneous implants at days 3 and 7. However, by day 21 the number of Cd68 positive cells increased in all implants regardless of Dex-loading. Scale bars = 500 µm.
Figure 40. Density of Macrophages within the 100 µm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in diabetic rats. Macrophage density increased with time for all implants regardless of treatment. Tissue surrounding Dex-releasing coatings had the lowest macrophage densities for all time points. Macrophage densities were on average lower in diabetic tissues when compared to healthy rats..... 163

Figure 41. Masson’s trichrome images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in Diabetic Rats. By day 21, the formation of organized collagen capsule was not observed on bare sensor implants. Scale bars = 200 µm........ 166

Figure 42. CD31 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in Diabetic Rats. No difference in vascularity between implants was observed for days 3, 7 and 21. Moreover, vascularity of diabetic tissue that surrounded implants was lower than the one presented in healthy rats. Scale bars = 500 µm......... 167

Figure 43. Density of blood vessels within the 100 µm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in healthy and diabetic rats at day 21 post-implantation. There was no differences between the vascularity around bare implants in healthy and diabetic rats. In diabetics, vascularity for all porous implants regardless of Dex-loading was lower when compared to healthy rat controls. ............. 168

Figure 44. Plot of sensor sensitivities of Medtronic MiniMed SOF-Sensor glucose sensors implanted in diabetic rats. Sensitivity of the sensors decreases after 7 days of implantation regardless of treatment................................................................. 172

Figure 45. Representative Glucose Bolus Tests of Medtronic MiniMed SOF-Sensor glucose sensors implanted in diabetic rats at (a) 1 Day, (b) 3 Days, (c) 7 Days, (d) 14 Days, and (f) 21 Days. Porous coated sensors did not respond to glucose challenges starting on day 14. All coated sensors regardless of Dex-loading had increased sensor lag when compared to controls. ................................................................. 173
Acknowledgements

I would like to thank Monty for bringing into the BME PhD program back in 2009. Monty, thank you for your continuous support, thinking about the big picture, keeping me on track, and the candid feedback. Most importantly, thank you for giving the freedom to develop my project and helping me grow into an independent scientist.

A big thanks goes to Dr. Bruce Klitzman for working with me through the bumps and obstacles the road to a PhD posed. Dr. K, you were an invaluable source of support and mentorship. Thank you for your positivity, encouragement, and advice.

I would also like to acknowledge Natalie Wisniewski for being an amazing role model and for insightful discussions about life and glucose sensor technology. Recognitions also go to Dr. Fan Yuan for teaching me about drug delivery and modeling, and to Dr. Howard Levinson for lending his clinical expertise to this project.

I would specially like to thank Brittany Davis. Though our projects never crossed paths, thank you for working alongside me for the past 6 years, pulling late nights in lab, and being my rock when I needed you most. You were the best coffee buddy I could have ever asked for!

Nga Brown taught me how to make rat jackets and showed me how fun animal work could be. Nga, thank you for taking care of the little dudes with me.

I would also like acknowledge the members of the Reichert, Klitzman, Truskey, and Yuan labs for their scientific input, companionship, and making me smile almost
every day. I would like to specially thank Mike Nichols, Alice Brochu, Mina Wu, Yvette Beben, Lucinda Camras, Tracy Cheung, Jinny Cho, Beth Lorden, Lizzy Mayorga, and Saniya Ali.

A big shout out goes to the folks that became my family outside of the lab. Devin, Tyler, Vrad, Pete, Marissa, Amanda, Nicky, and Paige – Thank you for all the trips and memories. I am looking forward to having many more adventures with you!

I thank my family for being an endless source of wisdom, love, and support. I specially thank my Mom for teaching me how to be a courageous woman and my Aunt for being a voice of reason. I also thank, my Sister and Cousin for being my partners in crime, my Dad for guiding me through my early years, and my Step-dad for the unwavering support that allowed me to pursue my goals.

Last, but definitely not least, I would like to thank Peter Faulkner for being my biggest fan. This past three years by your side have been the most exciting, daring, and fun. Thank you for your support, guidance, encouragement, and unconditional love. Thank you for believing in me, when I doubted myself most. I couldn’t have done it without you!
1. Chapter 1: Introduction

1.1. Clinical Significance

Type I diabetes is a chronic condition marked by high-glucose levels in the blood [1-4]. It affects millions of patients each year and is caused by either deficiency or tolerance to insulin [1, 2]. One of the most effective modes of treatment for this disease is a closed-loop system that can continuously monitor blood glucose levels and deliver insulin at adequate rates in response [3-6]. However, the development of such devices has been hindered by the untimely failure of glucose sensing platforms [4, 6-10]. This failure occurs due to an undesirable foreign body response to glucose sensor implantation [7, 10]. An aggressive initial inflammatory response followed by the formation of a fibrous capsule which is not associated nor supplied with blood vessels leads to erratic behavior and loss of signal at the sensor tip [7, 11]. Users will then have to repeatedly change sensors to attain optimal signal recordings and appropriate insulin delivery [8]. We believe that the functional life of glucose sensors could be extended by developing strategies that diminish inflammation, and promote tissue integration and blood vessel formation.
1.2. **Hypothesis**

Application of glucose sensors for long-term use is restricted by a wound-healing associated failure. Strategic localized drug delivery to attenuate early inflammation, in combination with the presentation of topographical cues to reduce fibrosis and promote vascularity will create a desirable tissue environment for working sensors. This new microenvironment will lead to long-term maintainable, reliable, and stable glucose sensor signals.

1.3. **Approach**

Inflammation and the formation of an avascular fibrous capsule have been identified as the key factors controlling the wound healing associated failure of implantable glucose sensors. We will guide tissue remodeling of implanted sensor leads by the temporal release of dexamethasone (Dex), a potent anti-inflammatory agent, in combination with the presentation of a stable textured surface. Dex will initially attenuate acute inflammation, while the porous textured surface will modulate the later stages of the response by increasing vessel density and reducing collagen deposition around indwelling sensor leads. A schematic of this strategy is provided in Figure 1.
Dex-releasing porous coatings of controlled pore size and thickness were fabricated using salt-leaching/gas-foaming technique. Porosity, pore size, thickness, Dex-release rate, drug loading amount, and drug bioactivity was also evaluated. In vitro sensor functionality test were performed to determine if Dex-releasing porous coatings interfered with sensor performance (increased signal attenuation and/or response times) compared to bare sensors.

The tissue modifying effects of Dex-releasing porous coatings were accessed by fully implanting them in the sub-cutaneous space of healthy and diabetic rats. Immunohistochemical techniques were be used to monitor the effects of these coatings on the tissue surrounding non-functional implants.

Finally, we deployed Dex-releasing porous coatings from the tips of functional sensors on healthy and diabetic rats to assess if their tissue modifying effects, translated into accurate, maintainable and reliable sensor signals. Sensor functionality was accessed by continuously monitoring glucose levels and performing acute glucose challenges at specified time points. Finally, immunohistochemical techniques were used to evaluate the tissue microenvironment of bare and coated functional sensors.
Figure 1. Schematic showing the effects of the foreign body response on sensors and the proposed Dex-releasing porous coating system.
2. Chapter 2: Specific Aims

2.1. Specific Aim 1: Fabrication and characterization of porous, dexamethasone-releasing coatings for implantable glucose sensors

Gas-foaming/salt-leaching technique was used to generate porous Dex-releasing polyurethane coatings ranging in porosity and drug loading. Various properties of the resulting coatings were investigated, including porosity, pore size, thickness, drug release rate, drug loading, drug state, and drug bioactivity. Once the coatings were fully characterized in vitro, sensor functionality test to coating presence were performed to determine the effects of coatings on sensor signal strength and response time.

Drug release from coatings monitored over two weeks presented an initial fast release followed by a slower release. Total release from coatings was highly dependent on initial drug loading amount. Functional in vitro testing of glucose sensors deployed with porous coatings against glucose standards demonstrated that highly porous coatings minimally affected signal strength and response rate. Bioactivity of the released drug was determined by monitoring Dex-mediated, dose-dependent apoptosis of human peripheral blood derived monocytes in culture. These results suggest that deploying
sensors with the porous, Dex-releasing coatings is a promising strategy to improve glucose sensor performance.

2.2. Specific Aim 2: Characterization of the foreign body response to porous, dexamethasone-releasing coatings in healthy and diabetic animal models

In order to examine the effects of Dex-releasing porous coatings on the tissue microenvironment of an indwelling sensor, coatings were subcutaneously implanted in the dorsum of healthy and diabetic rats. Implants of similar size, shape and mechanical properties to that of Medtronic MiniMed SOF-Sensor tips. Implants were either bare, or treated with Dex-free or Dex-releasing porous coatings. Implants were retrieved at days 3 and 7 to monitor the anti-inflammatory effects of coatings, and at days 14, and 21 to observe the tissue remodeling effects of coatings. Nonspecific and immunohistochemical tissue stains allowed for the qualitative and quantitative analysis of the tissue microenvironment.

Systemic unregulated glucose levels can cause altered wound healing and impaired immune function. Since glucose sensors are designed to be used by diabetic subjects, it is of interest to investigate the effects of dexamethasone release and topographical cues in a diabetic animal model. Diabetes was induced in healthy rats by
repeated intraperitoneal injections of streptozotocin (STZ). STZ is a toxic agent that targets the insulin-producing beta cells of the pancreas. A similar implantation procedure, sample size, and analysis was performed as described above for healthy rats.

In healthy subjects, histological observations and quantification showed that Dex-releasing porous coatings were capable of diminishing inflammation during early time points while promoting the formation of vascularized network of the tissue-sensor interface. Also, differences in wound healing patterns between healthy and diabetic subjects were observed; diabetic rats showed lower levels of inflammation and vascularization of the tissue surrounding implants when compared to their normoglycemic counterparts. These results suggest that deploying Dex-releasing porous coatings on the surface of sensors might be a promising strategy to remediate the biocompatibility-driven failure of implantable glucose sensors in healthy subjects.


To evaluate the effects of decreased inflammation and increased tissue perfusion on indwelling sensor function, modified functional glucose sensors were percutaneously implanted in the dorsum of healthy and diabetic rats. Sensors were implanted using an
in-house designed animal model that allowed for long-term awake animal measurements, and allowed for three sensors to be placed in a single subject. Implanted sensors were either bare, with porous coatings, or with Dex-releasing porous coatings. Implants were retrieved at days 3, 7 and 21 to monitor the anti-inflammatory effects, and to observe the tissue remodeling effects of coatings. Sensitivity measurements and glucose bolus tests were performed throughout the duration of the study as means of assessing sensor signal reliability, accuracy and responsiveness. Immunohistochemical tissue stains provided qualitative and quantitative analysis of the tissue microenvironment.

The tissue response to implants showed that sensors treated with porous Dex-loaded coatings diminished inflammation and enhanced vascularization of the tissue surrounding the implants. Functional sensors with Dex-loaded porous coatings showed enhanced sensor sensitivity over a 21-day period when compared to controls in healthy subjects. Enhanced sensor sensitivity was accompanied with an increase in sensor signal lag and MARD score. These results indicate that Dex-loaded porous coatings were able to elicit a favorable tissue response, and that such tissue microenvironment could be conducive towards extending the performance window of glucose sensors in vivo.
The diabetic pilot animal study showed differences in wound healing patterns between healthy and diabetic subjects; diabetic rats showed lower levels of inflammation and vascularization of the tissue surrounding implants when compared to their normoglycemic counterparts. Also, functional sensors with Dex-loaded porous coatings did not show enhanced sensor sensitivity over a 21-day period when compared to controls in diabetic subjects. Moreover, increased sensor signal lag and MARD score was present in coated sensors regardless of Dex-loading when compared to bare implants in diabetics. These results suggest that the altered wound healing patterns presented in diabetic tissues may lead to premature sensor failure when compared to sensors functioning in normoglycemic rats. Therefore, further investigation of the structural differences between diabetic and healthy cutaneous wound healing and how this difference affect the functional life of indwelling glucose sensors is needed.
3. Chapter 3: Background

3.1. Clinical Need

In the United States more than 25.8 million people are affected by either type I or type 2 diabetes [1, 2]. Diabetes is a degenerative disease characterized by patients that are unable to regulate systemic glucose levels [12]. This occurs either as a result of lack of insulin production by pancreatic β-cells or desensitization of insulin receptors in peripheral cells [1, 12].

Though, diabetes associated mortalities have decreased since the introduction of insulin therapy in the 1920s [13], most side effects such as increased risk of blindness, ketoacidosis, poor circulation, cardiovascular disease, and impaired wound healing remain highly unmanaged [2, 3]. These side effects are caused by long-term unregulated blood glucose levels [2-4]. Unfortunately, current diabetic management technologies such as finger-pricking and active insulin delivery do a poor job at achieving tight glycemic control [3-5, 14].

Thus, there is an increasing need for a system that can detect real time changes in glucose levels and deliver appropriate levels of insulin in response - the artificial pancreas [3-6]. The development of an artificial pancreas could allow for tight glycemic control and
prevent of hypo- and hyperglycemia episodes. Currently, the biggest limitation faced by artificial pancreas advancement is the design of an implantable continuous glucose sensor that can 1) provide reliable glucose readings for the delivery of insulin, and 2) survive in vivo over an extended period of time (> 5 days) [3, 9]. Hence, for the development of the artificial pancreas to be realized, issues surrounding the long-term in vivo sensor reliability must be resolved.

3.2. Tissue Response to Implantable Devices

The foreign body response (FBR) associated with glucose sensors involves protein absorption onto the sensor surface followed by cellular deposition, inflammation and formation of a fibrous capsule [10, 11, 15-19]. Figure 2 shows the time course of the FBR. A temporary fibrin protein matrix first forms and provides the basis for cellular infiltration and blood clothing. Platelets circulating in the blood will migrate to the site of injury to further prevent excessive bleeding. Neutrophils will be drawn, arriving within a few hours; they are short lived, fast migrating phagocytic cells. As they disappear, they will make way for more permanent inflammatory cells, macrophages. During inflammation, macrophages will tend to remain at the wound site for a few days to weeks. They will eat away foreign and cellular debris material. Within a few days to weeks post-
injury, a tissue intermediate composed of macrophages, fibroblasts cells, newly formed blood vessels and a more complex protein matrix (granulation tissue) will form. Even though somewhat vascular, this tissue will transform within 14-21 days of implantation to an increasingly avascular network mostly composed of collagen.

![Diagram showing time course of foreign body response to implantable device](image)

**Figure 2. Time Course of the Foreign Body Response to Implantable Device [19].**

### 3.3. In vivo Failure of Implantable Glucose Sensors and Glucose Transport Implications

In a series of pioneering studies, Peter Bungay and Paul Morrison from the National Institutes of Health utilized established physiological principles to analyze the mass-transport limitations of an analyte as it travels from the tissue into the dialysate of
an implanted microdialysis probe [20-23]. For the first time, these studies showed that reductions in microdialysis extraction from tissues was not caused by alterations to the probe’s membrane but rather produced by an increase tissue resistance. They hypothesized that increased resistance of the tissue was caused by augmented cellular metabolism, changes in the diffusion coefficient and volume fraction of the extracellular space, and altered microvascular exchange. Therefore, this seminal study established that micro-architecture implant-tissue interface has a significant effect on analyte transport.

Multiple reports cite the FBR as the main cause of premature sensor failure in vivo [7, 8, 10, 24, 25], and that the long-term survival and adequate functionality of a glucose sensor relies on its ability to elicit a favorable FBR [24, 26-28]. Upon implantation, a sensor is presented with a dynamic and harsh tissue microenvironment characterized by surface passivation, up regulation of proteolytic enzymes, metabolically active immune cells, increased fibrosis, and lack of vasculature [24, 25]. As a result, blood glucose measurements from an indwelling sensor are often erratic, attenuated, and unreliable [10, 29]. Figure 3 shows the stages of the FBR and how they affect sensor function and glucose transport to the sensor surface.
The initial deposition of blood plasma proteins and adhesion of platelets lead to fouling of the glucose sensor tip [10, 24]. In most occasions, the formation of this network will cause partial loss of signal of the analyte within only hours after implantation. If an indwelling sensors survives this initial period of intense cellular activity it will enter the so called “break-in” period. Here, the tissue surrounding the sensor will present inflammatory factors which lead to signal instability and the initial formation of granular tissue around the implant [11, 15]. Enzymatic attack and enhanced glucose consumption by metabolically active inflammatory cells will cause the sensor signal to become attenuated and erratic [25, 30]. At this point, sensors can either catastrophically fail due to impaired glucose readings or the signal can reach a steady state as a result of the stabilization of the tissue remodeling process. Within days, this granular tissue will transform into a collagen rich, fibrous, avascular structure, where signal readings are diminished due to sensor isolation from the surrounding network [11, 17, 18].

Mathematical modeling and experimental studies have recently identified inflammation and repair as the two key stages determining the fate of an indwelling sensor. Computational studies by Novak et al. showed that lack of vascularity and not collagen capsule thickness is the single most important factor that leads to sensor signal
attenuation [25]. However, in studies where a vascular microenvironment was created around implanted sensor leads, increased perfusion did not translate improved sensor performance and/or enhanced \textit{in vivo} durability [31-35]. It is believed that the unstable granular environment presented during inflammation masked the beneficial effects of increased perfusion causing erratic sensor behavior. Notably, \textit{in vitro} and \textit{in vivo} studies have also shown that metabolically active immune cells with enhanced glucose consumption may lead to sensor signal attenuation [30, 36-38].
Figure 3. Foreign Body Response leads to premature Glucose Sensor Failure.
One could then propose that a glucose sensing device will function best not only when presented with a well-perfused, vascularized tissue, but when the immune response to its implantation is also diminished; this will prevent early erratic behavior and promote stable readings in the long-term. Consequently, the goal should be to 1) take sensors past the unstable “break-in” period and 2) establish a well-vascularized, perfused tissue network around implanted sensor leads.

### 3.4. Biomaterial Strategies to Reduce Inflammation

Biomaterial-mediated inflammation is a complex reaction involving protein adsorption, leukocyte migration, localization and activation, and secretion of inflammatory mediators [11, 39]. The intensity of this response is largely dictated by the extent of tissue injury and surgical technique, implantation site, implant shape and size, and material chemical and physical properties [40]. Material strategies to mediate inflammation can be broadly categorized into either passive or active.

Passive strategies have involved preventing nonspecific protein adsorption and subsequent immune cell adhesion onto a biomaterial surface [10, 24, 40]. It is believed that preventing initial fouling can reduce ensuing leukocyte recruitment and tissue fibrosis [41, 42]. Though non-fouling coatings such as self-assembled monolayers [43], polymer
brushes [44, 45], and hydrogels [46] have proven effective at reducing protein adsorption and leukocyte in vitro, implementation of these coatings in vivo have not translated into reduction of acute and inflammatory responses [47].

Through the delivery the presentation or delivery of anti-inflammatory agents, active strategies present a more directed approach to regulate the tissue response. Cytokines [48], receptor antagonist [49], peptides [50], nitric oxide [26, 51] and glucocorticoids [39, 52] have been successfully immobilized or delivered from coating surfaces. These agents are capable of dampening the production of inflammatory cytokines and/or preventing migration of leukocytes to the injury site. The implementation of these strategies in vivo has been widely effective at controlling inflammation.

Dexamethasone (Dex) is a potent glucocorticoid associated with diminished emigration and activation of immune cells, up-regulation of anti-inflammatory cytokines, and decreased collagen production at the implant site [39, 53-55]. It can specifically modulate macrophage behavior and reduce the levels of inflammatory cytokines such as TNF-α [56], IL-1β [57], and Interferon-γ [58]. Owing to its stable cortisol structure, it is very stable and amicable to high-temperature processing [59]. However, when delivered
systemically, it can cause deleterious side effects such as reduction of bone mineral density, cataracts and skin thinning [60]. Moreover, long-term Dex delivery has been associated with dismissed tissue regeneration and neovessel formation after injury [32, 60].

Most notably, Dex delivery from coatings to mediate the foreign body response to percutaneous glucose sensors has been explored by the Burgess Group at the University of Connecticut. In series of publications, this group generated a coating composed Dex-loaded PLGA micro-particles embedded in a PVA hydrogel matrix [61-64]. Researchers showed Dex-releasing coatings were able to mitigate the inflammatory response to sensor implantation and that Dex delivery correlated with improved sensor signal and longevity. However, when most of the drug payload was depleted from coatings the beneficial effects of sensor coatings dissipated. The effects of these coatings was therefore transient and dependent on the Dex presence within the tissue surrounding the indwelling sensor. Hence, the use of anti-inflammatory agents as an exclusive mediator of FBR is not sufficient to permanently improve glucose sensor function.
3.5. *Tissue Response to Textured Surfaces*

Vascularization around an implanted sensor is key for promoting adequate long-term functionality [18, 25, 34, 35]. If functional blood vessels were to develop in direct apposition to the sensor lead, analytes could freely reach the device [65-67]. As first demonstrated by Davila in the 1960s, topographical cues delivered from implant surfaces have a profound effect on the nature of the foreign body response to an implanted device [68]. They observed that a large number of blood vessels formed around porous polymer sheets when compared to solid controls. Numerous recent findings have supported and extended this concept [31, 35, 65-67, 69, 70]. Bauker et al. also suggested that topography and not surface chemistry may control the tissue-material interactions that regulate the neovascularization process around implants [31]. Texturing has seen the greatest success when the size of the pores is within cellular dimensions (5 - 100 µm). These pores are sufficiently large to allow for cellular infiltration and tissue in-growth but small enough to disrupt fibrous tissue deposition.

Investigators have also noted that the collagen capsule that forms around porous materials is more loosely packed than the one observed in solid implants. A capsule that contains dense, tightly packed bundles of collagen is much more likely to hinder analyte
diffusion into a sensor than more loosely packed collagen. Sharkawy et al., showed that, as compared to solid implants, porous implants lead to a more sparse capsular tissue that resulted in enhanced fluorescein diffusion [66, 67]. The sparseness of the collagen may be due to the effect of pores on fibroblast function. As noted by Berry et al. specific features in substrate topography play an important role in the way that fibroblasts orient and attach to substrates [71].

Factors such as protein absorption, cellular attachment, orientation, and subsequent angiogenic factor secretion are drastically influenced by material topography [71-73]. One hypothesis is that porous materials create a state of chronic inflammation that prevents natural wound healing [74]. Immune cells at the implant site compete with fibroblast for a space within the porous gaps, this prevents the maturation of collagen inside of the porous material, and results in impaired collagen deposition and capsule formation. Most recently, it has also been suggested that macrophages within the pores secret cytokines that promote new blood vessel formation [75]. Macrophages in the pores may experience mechanically stress since they are not be able to fully spread onto the surface of the pores. As a result, macrophages will differentiate into an alternative phenotype (M2) that secrets pro-wound healing cytokines (VEGF and IL-10). Though
many hypotheses have come to the forefront, the exact mechanism through which porous materials disrupt fibrous capsule formation and promote new vessel growth has yet to be elucidated.

The use of porous materials to mediate the foreign body response to glucose sensors was first proposed by Updike et al. [76] and has been further studied by multiple research groups [18, 35, 63]. Koschwanez et al. was able to increase vascularization around implanted sensor leads by introducing highly porous PLLA coatings of controlled structure (50-75 μm pore size) [34, 35]. Though, increased vascularization to the sensor surface was reported, this effect did not correlate with improved sensor functionality. It is hypothesized that the unstable granular environment presented during inflammation masked the beneficial effects of increased perfusion leading to erratic sensor behavior. Therefore, enhanced vascularization without inflammation control has shown to not be sufficient to extend glucose sensor life.

### 3.6. Strategies to Improve In Vivo Glucose Sensor Biocompatibility

Strategies to extend sensor functionality focused on the preventing protein adsorption and cell attachment through the incorporation of hydrogel coatings, such as PEG [77] and PVA [55]. Current sentiment is that resistance to biofouling is a necessary
but not sufficient to ensure proper sensor function. Strategies for the attenuation of inflammation and the promotion of tissue vascularization have gained favor through the introduction of “tissue response modifiers”, such as the local release of growth factors (VEGF, PDGF), nitric oxide [26, 51], or anti-inflammatory glucocorticoids [39, 54, 55]. Topographical approaches have also been used to increase vascularization and promote tissue integration around implanted sensor leads [34, 35].

Table 1 summarizes recent strategies used to regulate the tissue response to implanted glucose sensors. To date, combined strategies to improve sensor biocompatibility have involved either dual agent release or antifouling gels merged with localized drug delivery. To our knowledge, researchers have yet to exploit the integration of drug release with topographical cues as an approach to locally modulate the tissue response to sensor implantation.

Table 1. Strategies to Mediate the Tissue Response to Glucose Sensor Implantation

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Material and Platform</th>
<th>Active Agent (Delivery Window)</th>
<th>Test subject/implant site/implant type</th>
<th>Length of Study</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attenuate inflammation</td>
<td>Rapid Release of small molecule donors</td>
<td>Nitric Oxide (1-3 days)</td>
<td>Rat/percutaneous/microdialysis probes</td>
<td>2 weeks</td>
<td>51</td>
</tr>
<tr>
<td>Function</td>
<td>Material/Technique</td>
<td>Animals</td>
<td>Duration</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
<td>---------</td>
<td>----------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Anti-fouling, attenuate inflammation</td>
<td>PVA Hydrogel and drug loaded PLGA microspheres</td>
<td>Rat/subcutaneous/stainless steel needle</td>
<td>4 weeks</td>
<td>55]</td>
<td></td>
</tr>
<tr>
<td>Attenuate inflammation, promote vascularization</td>
<td>pHEMA-PEG hydrogel and PLGA microspheres</td>
<td>Rat/percutaneous/microdialysis probes</td>
<td>6 weeks</td>
<td>32, 54]</td>
<td></td>
</tr>
<tr>
<td>Tissue biomimicry</td>
<td>Adipose Derived Stem Cells (ASCs) coatings</td>
<td>N/A</td>
<td>8 weeks</td>
<td>78]</td>
<td></td>
</tr>
<tr>
<td>Tissue biomimicry</td>
<td>Porous Collagen Coatings</td>
<td>N/A</td>
<td>4 weeks</td>
<td>79]</td>
<td></td>
</tr>
<tr>
<td>Promote tissue integration, vessel growth</td>
<td>Porous pHEMA and silicone scaffolds</td>
<td>N/A</td>
<td>6 months</td>
<td>80]</td>
<td></td>
</tr>
<tr>
<td>Promote tissue integration, vessel growth</td>
<td>Porous ePTFE coatings</td>
<td>N/A</td>
<td>3-6 months</td>
<td>76]</td>
<td></td>
</tr>
<tr>
<td>Promote tissue integration, vessel growth</td>
<td>Porous PLLA coatings</td>
<td>N/A</td>
<td>3 weeks</td>
<td>35]</td>
<td></td>
</tr>
</tbody>
</table>

### 3.7. Medtronic MiniMed SOF-SENSOR™ Glucose Sensors

Thanks to a generous donation from the Medtronic MiniMed we will using their Medtronic MiniMed SOF-SENSOR™ glucose sensor and MiniLink™ Wireless
Transmitter as a platform for our studies. This system relies on electrochemical detection of glucose through its reaction with glucose oxidase [5]. Figure 4 shows a schematic of the structure of this glucose sensing system. Glucose oxidase is immobilized on the surface of the working electrode and it catalyzes the oxidation of glucose into gluconic acid and hydrogen peroxide in the presence of oxygen. The constant voltage applied to the working electrode decomposes the generated hydrogen peroxide into oxygen, hydrogen ions and electrons. The generation of electrons, produces a current reading at the sensor tip. The produced current reading is proportional to the interstitial glucose concentration. 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.
Figure 4. Schematic of the Medtronic MiniLink™ System. The system is composed of a sensor that measures glucose levels via an electrochemical reactions. The current signal produced at the sensor tip can be transmitted wirelessly via an external transmitter to a computer or insulin pump [81].
Currently, the Medtronic MiniMed TM system is only for 3-5-day use in patients and is designed to be used as a complement conventional glucose monitoring technologies and not a as a replacement. Also, it is recommended that the sensor should be first calibrated within 2-4 hours post-implantation and every 12 hours for the remainder of its functional life via finger-pricking. These limitations are a result of the biocompatibility based failure of implantable glucose sensors. Therefore, there is a need for a platform that can extend the functional life of these devices.

3.8. Diabetic Wound Healing

Some of the most common side effects of diabetes are altered wound healing and higher susceptibility to infection [2, 82]. These undesirable healing patterns have been mostly studied within the context of foot ulcers and high rates of nosocomial infections after medical procedures [83]. Unfortunately, findings from these studies have yet to be applied to the understanding of a diabetic’s response to implants, such as implantable glucose sensors.

Upon injury, diabetic tissues may experience an impaired and/or delayed immune response as a result of immune cell dysfunction. Characteristically, diabetic wounds have shown decreased neutrophil and macrophage function, as well as decreased migration of
these cells to the tissue bed [84-87]. Neutrophils from diabetics have demonstrated reductions in functional activity contributing to the high susceptibility and severity of infections in diabetic patients [85]. Experimental studies in diabetic rats and mice have consistently reported defects on diabetic-neutrophil’s chemotactic, phagocytic, and microbicidal activities [88]. Functional studies of monocytes from type I diabetic patients have also showed an increase in secretion of IL-6 [89]. Moreover, hyperglycemia has been shown to alter tissue mast cell metabolism and inhibit their degranulation [90].

Dysfunctional inflammatory cell infiltration and metabolism have been linked to an altered expression of cytokines and chemokine’s in diabetic tissues [84, 91]. In diabetes there is a marked imbalance of pro-/anti-inflammatory cytokines which are released in a non-sequential manner. This leads to impaired tissue repair and weakened cellular and humoral immune response. Most notably diabetic wounds have shown decreased or untimely expression of TGF-β, MCP-1, Interferon-γ, and IL-8 all of which are known chemoattractants to neutrophils and macrophages [91, 92].

The alteration of the inflammatory response in diabetes is typically accompanied by systemic up-regulation of pro-inflammatory cytokines. Insulin resistant diabetes (Type II) has been associated with a low and steady release of pro-inflammatory
mediators such as IL-1, TNF-α, and IL-6 [93]. Moreover, acute hyperglycemic episodes in Type I diabetes induce the systemic production of IL-1, IL-4, and IL-6 [86]. Elevations on the levels of these cytokines can persist hours after the hyperglycemic episode has come to an end. Though diabetes leads to chronic systemic inflammation its translation into chronic peripheral inflammatory state that activates immune cells is still under question [93].

Aberrant cytokine release as a result of hyperglycemia, not only affects inflammation but tissue repair and remodeling. Later stages of the diabetic wound are characterized by persistence of inflammatory cells within the injured tissue which leads to continual tissue damage and turnover due to increased expression of metaloproteases (MMPs), TNF-alpha and IL-1β [83, 85, 87, 94]. Moreover, differential cytokine production also affects the functions of endothelial cells and fibroblasts. Poor vascularization of the tissues arises as a consequence of decreased levels of VEGF, PDGF, and epidermal growth factor at the site [91, 92, 94]. Diabetic patients show lower levels of circulating and wound EPCs [95]. Mobilization from the bone marrow to the circulation is impaired due to lower eNOS and NO production, while homing to the wound site is impaired due to low levels of SDF-1α expression at the wound site [91]. Moreover, increased superoxide production
at the wound site may lead to decreased nitric oxide bioavailability, which could cause dampened wound closure due to a reduction in collagen synthesis by fibroblasts [82, 85].

It is important to remark that the specific factors leading to an altered diabetic wound healing still remain under investigation [85, 91, 93, 94]. Though, it has been confirmed that there is a profound imbalance on the timing and secretion of cytokines, as well as impaired recruitment and functionality of the cells involved in tissue remodeling. Therefore, the diabetic tissue response to an indwelling sensor in diabetics may be significantly different than the one observed in normoglycemic tissues [94]. Since percutaneous glucose sensors are believed to undergo biocompatibility based failure, studying the diabetic wound microenvironment is necessary to further understand in vivo sensor failure, generate strategies to extend sensor functionality, and drive the rapid translation of glucose sensing technologies in to the clinical setting.

3.9. Diabetic Animal Models

Animal models have been key in the study of disease progression and complications of diabetes. Animal models of diabetes have been generated by either selective inbreeding for hyperglycemia, administration of toxins, single gene mutations, applications of transgenic techniques, and/or surgical removal of the pancreas[94]. Many
animal models exist that accurately portray the side effects, condition and disease progression of both Type I and Type II diabetes [96, 97]. Even though both result in hyperglycemia, they have very different causes, pathogenesis, and treatments. Diabetes Mellitus Type I is an autoimmune condition that results in the destruction of insulin producing β-cells in the pancreas [2, 12, 97]. Lack or low levels of insulin can lead to chronic hyperglycemia which can be controlled with supplemental insulin administration. In contrast, Type II diabetes is a metabolic disorder that is characterized by insulin resistance and/or altered levels of insulin production [12]. Type II diabetes can usually be managed by increasing exercise and dietary changes. Since patients suffering from insulin dependent diabetes will benefit the most from the development of a system that can continuously and accurately monitor glucose changes, examination of the FBR to glucose sensor should be first evaluated in a Type I diabetic animal model [5, 37, 42].

3.9.1 Spontaneous Models of Type I Diabetes

The Non-obese diabetic (NOD) mouse and Biobreeding (BB) Rat are both spontaneous animal models of diabetes which have been generated through selective inbreeding for hyperglycemia [94, 96, 98]. Diabetes in the NOD mice develops rapidly and spontaneously within 12-30 weeks of age. This model has been used extensively in
diabetes research because of its similarity to the onset and immune pathology of the human condition. However, NOD mice generally present much milder ketoacidosis than the one observed in humans. Studies in the NOD model have focused on cell metabolism in diabetic wounds and immune cell functionality studies [94]. Similarly, to the NOD mice in the BB Rat model inflammation of pancreatic β-cells induces the autoimmune response that results in the destruction of the pancreas. BB diabetic rats incur on weight loss, hyperglycemia, and other diabetic complications which ensue within 12 weeks of age. Ketoacidosis in this model is more severe and fatal than in humans.

3.9.2 Chemically Induced Models of Type I Diabetes

The effects of systemic hyperglycemia can be most easily studied in animals by partial or complete damage of the pancreas [96-98]. This can be achieved either by surgical removal of the pancreas or delivery of toxins that impair pancreatic function. A single large dose or multiple small dosages of streptozotocin (STZ ) have demonstrated to produce diabetes in rodents [84]. Streptozotocin is a naturally occurring toxic agent that can selectively destroy the insulin-producing β-cells of the pancreas. It is toxic to cells by causing DNA damage. B-cells have relatively high levels of glucose transport protein GLUT2. STZ’s is structure is similar enough to glucose that it is recognized and
transported into the cell exclusively by GLUT2 [99]. This model is particularly useful at studying complications of as a result of hyperglycemia. It produces many of the signs and symptoms of chronic human diabetes, such as, a diastolic cardiac dysfunction, cataracts and neuropathy [96, 100]. Wound healing studies in this animal model showed that genetically healthy rats when made by hyperglycemic by STZ administration heal less effectively than controls [84]. STZ diabetic animals from this study also experienced greater propensity to infection and decreased collagen production at that the wound site than controls.

For our investigations, the STZ diabetic animal model was used to study the FBR to glucose sensor implantation. Nevertheless, it must be noted that a single animal model may not accurately represent the complete pathophysiology of the human condition [96-98]. Thus, glucose sensor implantation studies in multiple animal models of diabetes may be necessary to fully evaluate the effects of diabetic wound healing on glucose sensor function.
4. Chapter 4: Specific Aim 1 - Fabrication and characterization of porous, dexamethasone-releasing coatings for implantable glucose sensors

4.1. Synopsis:

Commercially available implantable needle-type glucose sensors for diabetes management are robust analytically but can be unreliable clinically due to tissue-sensor interactions. This chapter presents the physical, drug release, and bioactivity characterization of tubular, porous dexamethasone (Dex) releasing polyurethane coatings designed to attenuate local inflammation and promote vascularization in the tissue-sensor interface. Porous polyurethane coatings were produced by the salt-leaching/gas-foaming method. Scanning electron microscopy (SEM) and Micro-computed tomography (Micro-CT) showed a controlled porosity and coating thickness. *In vitro* drug release from coatings monitored over two weeks presented an initial fast release followed by a slower release. Total release from coatings was highly dependent on initial drug loading amount. Functional *in vitro* testing of glucose sensors deployed with porous coatings against glucose standards demonstrated that highly porous coatings minimally affected signal strength and response rate. Bioactivity of the released drug was determined by monitoring Dex-mediated, dose-dependent apoptosis of human peripheral blood derived
monocytes in culture. These results suggest that deploying sensors with the porous, Dex-releasing coatings is a promising strategy to improve glucose sensor performance.

4.2. Introduction:

The dominant management strategy for blood sugar control in type I diabetes mellitus is the combination of blood glucose monitoring by finger pricking and manual insulin delivery. These strategies are often inadequate in cases where tight glycemic control is prescribed [3-5, 14]. Consequently, there is a pressing need for a closed loop system where real-time changes in glucose levels monitored by a sensor are used to regulate automated insulin delivery [3-6]. Unfortunately, contemporary implantable needle-type glucose sensors that could be used to manage insulin supply can behave unpredictably in vivo.

Often, a sensor that performs robustly and accurately in vitro may upon implantation continue to work adequately, fail acutely, show a steady drift, or exhibit a combination thereof. Interestingly, upon post-removal testing, the sensor will regain proper functionality [7, 9, 24]. This observation suggests that the unpredictable behavior of implanted sensors may be driven by the tissue-sensor interaction and not by failures in the sensor itself.
Implanted glucose sensors are subject to a dramatically varying tissue microenvironment over the 5-7 days that they are approved for patient use. Upon implantation, the sensor is presented with hemostasis followed by immune cell recruitment and inflammation, and finally the tissue gives way to a repair/remodeling stage comprised of provisional matrix formation, fibrosis, and loss of vasculature. Several excellent reviews are available on this topic [11, 24]. Adequately surviving this sequela of events, often referred to as the break-in period, has become an important design criterion in the development of implantable glucose sensors.

Initial strategies to extend sensor functionality have focused on the preventing protein adsorption and cell attachment through the incorporation of hydrogel coatings [55, 77]. The emerging sentiment is that resistance to biofouling is necessary but not sufficient to ensure proper sensor function. Numerical modeling and in vitro studies have recently shown that increased glucose consumption and enzymatic attack by immune cells during inflammation may be one of the dominant factors negatively affecting glucose sensor function [30, 36, 38].

Currently there exist essentially two schools of thought towards addressing acute inflammation: management and attenuation. Proponents of inflammation management
view inflammation as a necessary step to achieve a stable and acceptable tissue bed for an implanted glucose sensor. Approaches that manage acute inflammation are inherently more complex and employ strategies to guide immune cell phenotype and cytokine production \[101, 102\]. Attenuation of inflammation contends that the benefits of minimizing the deleterious effects of acute inflammation on sensor function outweigh the potential advantages of engineering the tissue response. One strategy for the attenuation of acute inflammation involves the local release of anti-inflammatory mediators such as nitric oxide, non-steroidal anti-inflammatory drugs, and glucocorticoids \[28, 51, 103\].

Recent reports have shown that localized delivery of dexamethasone (Dex) reduces anomalous sensor effects that arise from inflammatory cell invasion to the surface of an indwelling sensor \(20\). Dex is a potent glucocorticoid associated with diminished activation of immune cells and up-regulation of anti-inflammatory cytokines \(11, 21-24\). However, localized delivery of Dex is often accompanied by decreased vascularity at the sensor-tissue interface \(25\). In previous studies have also demonstrated that highly porous coatings of controlled structure \(50-75 \mu m\) pore size) could be used to increase vascular perfusion of the tissue bed \(26, 27\); to our knowledge researchers have
yet to explore the combination of these to proven effects as a strategy to improve indwelling glucose sensor function.

Our goal is to incorporate Dex-release as an inflammation attenuation component into a textured coating designed to increase long-term vascular density around implanted sensor leads. Therefore, the current study looked at the possibility of combining proangiogenic texturing with anti-inflammatory Dex release.

Here, we present the fabrication and characterization of Dex-releasing porous polyurethane coatings for needle type glucose sensors. Pore size, porosity, and coating thickness of the porous polyurethane coatings were evaluated by scanning electron microscopy (SEM) and Micro-computed tomography (Micro-CT). Signal strength and response time of sensors deployed with porous coatings was demonstrated using glucose standards. Drug release from porous coatings was monitored over two weeks as a function of initial loading and coating porosity. Bioactivity of the released drug was demonstrated by monitoring Dex-mediated, dose-dependent apoptosis of human peripheral blood derived monocytes in culture. These results suggest that deploying sensors with the porous, Dex-releasing coatings is a promising strategy to improve glucose sensor performance.
4.3. Materials and Methods:

4.3.1. Fabrication of Dex-Releasing Porous Coatings

Porous coatings were fabricated by the gas-foaming/salt-leaching technique described previously [35]. Briefly, a 6.5 wt % solution of polyurethane was prepared by dissolving Tecoflex® 93A pellets (Lubrizol, Technologies) in a solution of 25:75 ethanol to chloroform ratio. Dex (Sigma, D1756) was dissolved in the polymer solution and stirred until clear. Sieved (50-75μm) ammonium bicarbonate salt particles (MP Biomedicals, 150107) were added to the polymer solution and homogeneously mixed Figure 5. Table 2 lists the amount of Dex and ammonium bicarbonate porogen added to polyurethane stock solutions to produce various specimen compositions. Dex-free porous and non-porous coatings were fabricated either by respectively not adding Dex or ammonium bicarbonate porogen to the polymer solution. The fabrication procedure is illustrated in detail on Figures 5 and 6.

Table 2. Porogen and Dex added to stock polymer solution to produce coatings different porosities and Dex loadings

<table>
<thead>
<tr>
<th>Intended % Porosity</th>
<th>Porogen (g)</th>
<th>Intended wt % Dex</th>
<th>Dex (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>1.5</td>
<td>2.9</td>
<td>180</td>
</tr>
<tr>
<td>60</td>
<td>1.0</td>
<td>1.4</td>
<td>90</td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
<td>0.7</td>
<td>45</td>
</tr>
</tbody>
</table>
Figure 5. Fabrication of Dexamethasone-Releasing Porous Coatings.
Figure 6. Gas-Foaming/ Salt-leaching produces was employed to generate an interconnected pore network in dexamethasone-releasing porous coatings.
Polymer films were dip-coated onto copper wire mandrels (Belden, 20 AWG) and allowed to dry for 1 h. Films were porated by placing the polymer coated mandrels into DI water for 5 min at 90 °C to allow gas-foaming/salt-leaching to occur. Porated films were then quickly quenched in 4 °C deionized water for 20 min. Films were allowed to dry in over-night in a desiccator, and cut to a length of 1.5 cm in order to fit over the sensing tips of Medtronic MiniMed SOF-SENSOR™ sensors Figure 6 and Figure 7A.
Figure 7. Pictures and ESEM images of porous coatings created via salt leaching/gas-foaming technique. (A) Picture of Medtronic MiniMed Sof-Sensor™ with and without highly porous Dex-releasing coatings. (B) ESEM of exterior surface and (C) cross-section of 90% Porosity – 2.9 wt % Dex coatings. Arrows indicate pore diameters (B) and coating thickness (C). (n=6)
4.3.2. Scanning Electron Microscopy (SEM)

A vacuum sputter coater (Denton Desk IV) was used to deposit a 10 nm gold on the surface of the porous coatings. SEM images of the porous coating surfaces and cross-sections were analyzed using ImageJ. A length measurement tool was generated based on the ratio of pixels per scale bar length of each image. Images were standardized to cover a 0.5 mm² area of the coatings. Using the measurement tool, the major axis of each pore on the selected surface was measured (Figure 7B). Cross-sectional images were used to measure the thickness of the coatings at the positions highlighted (Figure 7C).

4.3.3. Micro-Computed Tomography (Micro-CT))

Porous coatings were soaked in Lugol’s Iodine (EMS Cat. #26055) for 3 days and were vacuum dried overnight. Samples were evaluated using the Nikon XTH 225 ST Micro-CT scanner. The X-ray source was set to 80 kV and 120 μA, spot size of < 3 μm, and a rotation step of 0.5 °. An exposure time of 708 ms was set for each X-ray image. Four X-ray images were then averaged to obtain one 2D projection. After acquisition, 2D projections were reconstructed using CT Agent software to provide axial picture cross-sections. After reconstruction, the data was converted into 2000 16-bit picture files with a resolution of < 3 μm/pixel. Complete volumes were rendered in Avizo Fire 8.0. Sub-
volumes consisting of 500 z-stack images were selected for porosity evaluation. Sample porosity was calculated using void to solid volumes. Percent porosity was calculated based on the following formula:

\[
% \text{Porosity} = \frac{\text{Void Volume}_{\text{Coating} i}}{\text{Void Volume}_{\text{Coating} i} + \text{Solid Volume}_{\text{Coating} i}} \times 100\%
\]

4.3.4. Differential Scanning Calorimetry (DSC)

DSC (PerkinElmer, Diamond DSC N5360020) was employed to determine the physical state of Dex in the polyurethane coatings. Pre-weighed 10 mg samples of Dex-loaded porous coatings were placed into aluminum pans and loaded into the sample chamber. Untreated Dex powder (Sigma, D1756) samples were used as controls. Control samples weighted 0.5 mg to match the amount of Dex in contained in the 10 mg Dex-loaded porous coating samples. The sample chamber was purged with nitrogen to prevent air oxidation of the samples at high temperatures. Samples were kept at 50°C for 10 min during preheating, and then samples were heated a constant rate of 10°C/min until a temperature of 400°C was reached. Degree of Dex crystallinity was determined by measuring Dex’s characteristic endothermic peak at 269°C.
4.3.5. High Performance Liquid Chromatography (HPLC)

Dex solution concentrations were determined using HPLC (Waters 2690) and a dual absorbance UV detector (Waters 2487). The isocratic mobile phase consisted of 42% acetonitrile and 58% water with a flow rate of 1 ml/min. Samples of 50 μl were injected directly and pumped through an Omnisphere 5 C18 Column. Dex was detected using a UV detector at 246 nm. The retention time of dexamethasone was 3.7 min. Dex solution concentration was determined from a standard curve of Dex in PBS from 1 to 100 μg/ml.

4.3.6. Dexamethasone Release Studies

Coatings were placed into micro-centrifuge tubes containing 1.5 ml of PBS (pH 7.4) at 37°C. Coatings were moved into fresh 1.5 ml PBS solutions every 24 h for a period two weeks. Daily drug release samples were stored at 4°C in the dark until analysis. Samples were thawed at room temperature and injected into the HPLC and the Dex concentration was determined as described above.

To evaluate possible topographical changes due to the depletion of Dex, coatings before and after the two-week release study were imaged by ESEM (wet mode). Captured images were processed as described above to calculate average thickness and pore size.
4.3.7. Dexamethasone Loading Efficiency

Pre- and post- gas-foamed coatings, and coatings after drug release studies were completely dissolved in 1 ml chloroform. Solutions were then injected into the HPLC and the Dex concentration was determined as described above.

4.3.8. Sensor Response

Medtronic MiniMed SOF-SENSOR™ glucose sensors, MiniLink™ transmitters, and Transmitter Utility software package were graciously supplied by Medtronic MiniMed (Northridge, CA). Bare sensors were hydrated for 2 h in PBS (37°C, stirred) and then dipped sequentially in 100, 200, 400, and 0 mg/dl glucose in PBS (37°C, stirred) to obtain a calibrated baseline sensor response. Dex-loaded porous coatings were subsequently placed over the sensor tips and subjected to a glucose challenge with 100, 200, 400, and 0 mg/dl of glucose. Dex-free non-porous films served as controls.

Sensor response time, and signal attenuation to glucose challenge were used as metrics to evaluate sensor function. Sensor response times were calculated as the time for the sensor to achieve 90% of its steady state calibration current for a given test interval. Attenuation was calculated as one minus the ratio of the peak sensor current during a
given glucose challenge interval divided by the sensor current over the same interval during calibration.

\[
\text{Attenuation\%} = \left(1 - \frac{\text{Sensor Current}_{\text{Challenge Interval}}}{\text{Sensor Current}_{\text{Calibration Interval}}} \right) \times 100\%
\]

4.3.9. Peripheral Blood Derived Human Monocyte Isolation

Human monocytes were isolated from 100 ml of EDTA-treated blood drawn from healthy volunteers (n=12). Using a hypotonic density centrifugation method, buffy coats were collected from the interphase of Histopaque 1077® (Sigma, 10771). After separation, mononuclear cells were washed twice with complete RPMI 1640 medium. Subsequently, untouched monocytes were negatively selected using magnetic beads (Dynabeads®, Life Technologies). Enriched Monocytes were then incubated in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated AB serum, 50 μm 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM Hepes, 4.5 g/L glucose, and 1% antibiotic/antimycotic solution. All cultured reagents used had endotoxin levels of < 0.01 ng/ml LPS. The isolated monocyte suspension was adjusted to a concentration of 1x10^6 cells/ml. The viability of the monocytes was > 95% as determined by Calcein AM intracellular stain and purity was > 90% as assessed by CD14+ marker expression using flow cytometric analysis.
4.3.10. Dex-treated Media Preparations for Apoptosis Assays

Two apoptosis studies were conducted to (1) investigate if Dex released from porous coatings retained its bioactivity after gas-foaming/salt-leaching, and (2) to show the dose-dependent effect of Dex released from porous coatings on isolated monocytes.

For Dex bioactivity studies, 90% porous coatings loaded with 1.4 wt % Dex were placed in RPMI for 24 h at 37 °C allowing Dex to be release into the media, the coatings were then moved to a second set of fresh media for another 24 h, and then to a third set of fresh media for another 24 h. This yielded media samples with Dex released from coatings after 24, 48 and 72 h. Dex concentrations from these solutions were determined by HPLC. Equivalent “Dex-spiked media” solutions were made by spiking RPMI media with concentrations equal to that of the Dex released from 90% porous coatings loaded with 1.4 wt% Dex at 24, 48 and 72 h. Untreated media and media treated with Dex-free porous coatings served as controls.

For Dex dose-dependent studies, 90% porous coatings loaded with 0.7, 1.4 and 2.9 wt% Dex were incubated in RPMI media as described above for 24, 48, and 72 h. These solutions were then used to culture isolated monocytes. Untreated media and Dex-free porous coatings served as controls.
4.3.11. Annexin-V Apoptosis Assays

For both Dex apoptosis studies, monocytes were re-suspended to a density of 1x10^6 cells/ml in treated and untreated media immediately after isolation. Cells were plated in 6-well tissue culture plates. Cell samples from each well were collected every 24 h for a period of 3 days and cultured at 37°C under 5% CO2. During sample collection, cell medium was replaced by suspending monocytes to a concentration of 1x10^6 cells/ml concentration in new treated or untreated media respectively.

Up-regulation of phosphatidylserine (PS) receptor expression was detected via Annexin-V antibody staining in treated peripheral blood derived human monocytes. For staining, samples were kept in ice washed twice in staining medium (ice cold PBS supplemented with 10% FBS and 1% Sodium Azide). Monocytes were directly labeled with APC-mouse mAb to CD14+ (Invitrogen, MHCD1405) and Annexin-V FITC to PS (Invitrogen, A13199). APC and FITC-conjugated murine IgG mAbs of unrelated specificities were used as negative stain controls. Annexin-V positive controls were created by treating U937 cells with camptothecin at a concentration of 4 μg/ml for 4 h. After staining, suspended cells were kept in ice and protected from light until Annexin-V and CD14+ expression was analyzed by flow cytometry analysis.
4.3.12. Statistical Analysis

All data are presented as means ± standard errors of the mean (±SEM). Coating pore size, thickness, and porosity results were compared using a one-way ANOVA followed by Tukey’s HSD tests for all samples (p < 0.05). Sensor functionality and drug release were analyzed using two-way repeated measures ANOVA followed by Tukey’s HSD post-hoc test (p < 0.05). Annexin-V Apoptosis assay results were analyzed using a two-way repeated measures ANOVA. Lilliefors test on apoptosis data confirmed data normality while a Maulchy correction was performed since the data violated Maulchy’s sphericity test (unequal variances). Tukey’s HSD was used for post-hoc tests and the threshold for significance was p < 0.05.

4.4. Results:

4.4.1. Coating Pore Size, Thickness and Porosity

Figure 7A shows a bare glucose sensor (left) and a sensor with a 90% porous coating (right). Coatings fit snugly over the sensor tip and were porous throughout. Figure 7B and Figure 7C are SEM images showing the porous microstructure and coating cross-section. Figure 8 contains Micro-CT volume renderings of the solid (A-D) and corresponding void (E-H) spaces of a Dex-free 90% porous coating, and Dex-loaded 90%,
60%, and 30% porosity coatings. Total void volume and interconnected void regions represented by individual colors in Figure 8 E-H clearly diminished with decreasing porosity.

Table 3 lists the average coating thickness and pore size measured by SEM and the coating percent porosity calculated by Micro-CT for specimens that were intended to generate porosities of 90%, 60% and 30%. A Dex-free 90% porosity specimen was included for comparison. Coating thickness increased significantly with decreasing porogen content, but no statistical difference in average pore diameter was found across these specimen types. The average percent porosity measured by Micro-CT closely mirrored the intended specimen porosities of 90%, 60% and 30%. The inclusion of Dex at the highest 2.9 wt% loading did not significantly affect pore structure, porosity, or coating thickness when compared to porous Dex-free coatings.
Figure 8. Representative micro-CT images of porous coatings created via salt leaching/gas-foaming technique with decreasing porogen fraction. Coatings of different morphologies were created by varying the ammonium bicarbonate porogen concentrations of (A-B) 90%, (C) 60%, and (D) 30% are shown. Addition of Dex did not disrupt scaffold structure of (A) 0 wt % Dex and (B-D) 2.9 wt %. Corresponding 3D volume renderings of porous structures are also shown, individual colors represent interconnected void regions of coatings (E-H). (n=6)
Table 3. Physical Characteristics of Porous Dex-releasing Coatings (n=6)

<table>
<thead>
<tr>
<th>Intended Composition</th>
<th>Thickness (µm)</th>
<th>Pore Diameter (µm)</th>
<th>Measured Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porosity %</td>
<td>Dex (%wt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0</td>
<td>85.3 ± 7.5</td>
<td>75.8 ± 10.1</td>
</tr>
<tr>
<td>90</td>
<td>2.9</td>
<td>80.6 ± 8.3</td>
<td>70.4 ± 13.5</td>
</tr>
<tr>
<td>60</td>
<td>2.9</td>
<td>130.5 ± 9.3*</td>
<td>76.8 ± 12.3</td>
</tr>
<tr>
<td>30</td>
<td>2.9</td>
<td>205.9 ± 17.9#</td>
<td>72.8 ± 14.9</td>
</tr>
</tbody>
</table>

* denotes p<0.05 or less compared to 90 % Porosity – 2.9 wt% Dex Coatings
# denotes p<0.05 or less compared to 60 % Porosity – 2.9 wt% Dex Coatings

4.4.2. Differential Scanning Calorimetry

DSC was employed to determine whether Dex loaded into coatings was present in a molecularly dissolved state or was sequestered as crystals. The dashed lines in Figure 9A and Figure 9B show the characteristic exothermic melting of bulk Dex crystals (dashed lines) at 269°C. This peak was absent in all Dex-loaded polymer coatings (solid and dotted lines). These results suggest that Dex was highly soluble in the polyurethane matrix and was not sequestered in a crystallized form.

4.4.3. Dexamethasone Loading and Retention after Gas-Foaming

Table 4 lists the Dex loading before (intended) and after the salt-leaching/gas-foaming step (retained) for the coating formulations used in Dex release studies. The wt% Dex retained after gas-foaming correlated directly to the initial Dex loading, while the
Figure 9. Differential Scanning Thermographs of dexamethasone loaded porous coatings. Dex is found in an amorphous state within the scaffolds. (A) Initial loading amounts of Dex were increased from 0.7-2.9 wt % Dex. Dex is found in a similar crystal states in scaffolds with high (2.9 wt %) and low (0.7 wt %) initial loading amounts. Actual loading amounts are listed in Table 3. (B) Changes in coating porosity did not affect crystal state of Dex within scaffolds. (n=6)
Table 4. Loading efficiency and release summary of Dex incorporated into porous polyurethane coatings by salt-leaching/gas-foaming method. (n=10)

<table>
<thead>
<tr>
<th>Intended Sample Composition</th>
<th>Dex retained after foaming</th>
<th>Average Release Rate (µg/day)</th>
<th>Dex retained after 14 day release (%wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Porosity %</td>
<td>Dex (%wt)</td>
<td>%wt</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------------</td>
<td>-----------</td>
<td>-----</td>
</tr>
<tr>
<td>90</td>
<td>0.7</td>
<td>0.56 ± 0.07</td>
<td>305.1 ± 35.3</td>
</tr>
<tr>
<td>90</td>
<td>1.4</td>
<td>1.07 ± 0.09*</td>
<td>554.5 ± 46.2*</td>
</tr>
<tr>
<td>90</td>
<td>2.9</td>
<td>2.10 ± 0.38*#</td>
<td>914.3 ± 162.9*#</td>
</tr>
<tr>
<td>60</td>
<td>2.9</td>
<td>2.41 ± 0.30</td>
<td>1195.2 ± 149.4</td>
</tr>
<tr>
<td>30</td>
<td>2.9</td>
<td>2.51 ± 0.32</td>
<td>1790.5 ± 229.5</td>
</tr>
</tbody>
</table>

* denotes p<0.05 or less compared to 0.7 wt% Dex – 90% Porosity Coatings
# denotes p<0.05 or less compared to 1.4 wt% Dex – 90% Porosity Coatings
percent Dex retained after the gas-foaming/salt-leaching step for all groups averaged 79.7± 2.5% (Mean ± SEM).

4.4.4. Dexamethasone Release

Figure 10 presents the two-week in vitro release profile of Dex from (A) 90% porous coatings prepared with 0.7, 1.4 and 2.9 wt% Dex loadings, and (B) 90%, 60% and 30% porous coatings prepared with 2.9 wt% Dex loadings. Overall, the cumulative Dex release increased significantly with the amount of Dex payload. In each case, a fast release rate was observed in Days 1-7, followed by a slow release rate in Days 8-15 (Table 4). Release rates from days 1-7 increased with increasing Dex payload, either by increasing the wt% Dex for a constant % porosity (Figure 10A), or by decreasing the % porosity for a constant wt% Dex (Figure 10B). Second-week release rates also followed the same trend but the effect was less pronounced and not significant. The last column in Table 4 lists the wt% Dex that remained in the coatings after 14 days of release. The percent retained after 14 days averaged 16.0± 0.9% (Mean ± SEM) over all specimens, again bearing no relationship to percent porosity. Moreover, SEM imaging of 90% porous coatings before and after 14 days of Dex release showed the coating thickness and porosity to be unaltered.
(data not shown), which one would expect for solubilized small molecule release from a non-degradable polymer.
Figure 10. Cumulative release of dexamethasone from porous coatings. (A) Initial loading amounts of dexamethasone (0.7, 1.4 and 2.9 wt %) and (B) original porogen content (90, 60 and 30% Porosity) were varied. Dexamethasone release from coatings shows high dependency on initial loading. * denotes p<0.05 or less compared to highlighted treatments (n=10)
4.4.5. Sensor Response Time and Signal Attenuation

Figure 11A and Figure 11B are the *in vitro* responses of bare sensors and sensors fitted with porous coatings to step increases in glucose concentration. Error! Reference source not found. lists the corresponding sensor response times and percent attenuations. All signals for sensors deployed with 90% coatings were superimposed directly over the bare sensor traces, and exhibited only slight increases in response times and signal attenuation regardless of wt% Dex loading. However, sensors fitted with non-porous coatings, 60% and 30% porosity films showed significantly increased response times and signal attenuation compared to base sensors. Clearly, the 90% porous coatings were best suited for sensor deployment.
Figure 11. Response of sensors with scaffolds of (A) varying porosities and (B) varying Dex loading concentrations compared to the response of bare sensors (without coatings) and sensors with non-porous coatings. Sensors were subjected to a glucose challenge by exposing them to a series of glucose concentrations in the following order: 0 mg/dL, 100 mg/dL, 200 mg/dL, 400 mg/dL and 0 mg/dL in PBS at 45 min intervals at 37 °C in stirred conditions. Decreasing coatings porosity resulted in increased in sensor response time and attenuation, while changes in Dex-loading did not affect sensor signal when compared to bare sensors. Data presented are representative traces of 6 independent tests. (n=6)
Table 5. In vitro response of sensors deployed with Dex-releasing polyurethane porous coatings. (n=6)

<table>
<thead>
<tr>
<th>Sample Composition</th>
<th>Time (min) to Reach 90% Steady State Current</th>
<th>% Attenuation 400 → 0a Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Porosity %</td>
<td>Dex (wt %)</td>
</tr>
<tr>
<td>Bare sensor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.9</td>
<td>&gt;45b*#&amp;   &gt;45b*#</td>
</tr>
<tr>
<td>Non-Porous Coating</td>
<td></td>
<td>&gt;45b*#&amp;   &gt;45b*#</td>
</tr>
</tbody>
</table>

a Glucose challenge (mg/dL)
b Time exceeded experimental interval
* denotes p<0.05 or less compared to Bare Sensor
& denotes p<0.05 or less compared to 60% Porosity -2.9 wt% Dex Coating
4.4.6. Bioactivity of Dex-Releasing Porous Coatings

Figure 12 displays the percent of Annexin-V positive (i.e. apoptotic) monocytes after being exposed to Dex-releasing 90% porous coatings for 24, 48 and 72 hours. The 2.9 wt% Dex coatings significantly showed the highest percent of Annexin-V positive monocytes at 24, 48 and 72 h, with 25.5%, 49.34% and 65.5% cells being apoptotic, respectively. A significant dose dependence of wt% Dex at 48 and 72 h was observed. Apoptosis of monocytes cultured in media treated with Dex-free porous coatings was similar to the baseline level of apoptosis in untreated media. Finally, identical assays performed with Dex-spiked media exhibited equivalent levels of apoptosis as those using media from Dex released for coatings, showing that gas foaming step had no effect on Dex bioactivity (data not shown).
Figure 12. Dose Response of Human Peripheral Blood Derived Monocytes to Dex released from porous coatings. Monocytes were incubated in medium treated with porous coatings ranging from 0.7-2.9 wt % in initial Dex loading. Dex released from scaffolds induced apoptosis of monocytes in a dose and time dependent manner. There was a strong dose dependency on monocyte apoptosis and initial Dex coating loading after 48 and 72 hrs of incubation. *denotes P < 0.05 or less compared to untreated medium (n = 8).
4.5 Discussion:

Tissue-associated anomalies with implanted glucose sensors have been an area of concern for many years. These effects can begin immediately following implantation or can arise during the “break-in period” that occurs during the first few days following sensor insertion. During this time, the sensor is presented with an unstable microenvironment characterized by immune cell infiltration, inflammation, and formation of a granulation tissue. Strategies for improving in vivo sensor performance development of new biomaterials and localized drug delivery for resisting biofouling, attenuating inflammation, and increasing vascularization of the foreign body capsule [25, 32, 34, 51].

Previously, we reported porous polyester coatings deployed at the tips of needle-type glucose sensors that increased vascularization and perfusion of the tissue-sensor interface; however implanted sensors succumbed to inflammation and immune cell infiltration resulting in early and pronounced signal reduction [35]. We now employ a segmented polyurethane in order to release the moderately hydrophobic anti-inflammatory glucocorticoid dexamethasone (Dex) from the porous coatings. The present study characterized the physical properties and drug releasing properties of the porous
coatings, the bioactivity of the Dex released from porous coatings, and the effect of the deployed porous coatings on sensor performance.

Porous Dex-loaded Tecoflex® 93A coatings had a tunable microstructure. Pore size was controlled by sieving of ammonium bicarbonate particulates to a desired size range of 50-75 μm. Immersing particulate containing polymer coatings in a heated water bath generated pores with sizes in the upper-end of the sieved salt particle size range (Table 2). Micro-CT generated void volume renderings showed a mostly interconnected pore structure that was most pronounced in coatings with high porogen content (Figure 8). Moore et al. similarly demonstrated that increasing the concentration of leachable sodium chloride particulates in polyester scaffolds for bone tissue engineering resulted in increased scaffold void volume and pore interconnectivity [104, 105].

The poration step caused between 13% and 28% of the originally loaded Dex to be lost from the polymer coating (Table 4). The largest Dex loss occurred for the specimens with the highest polymer surface area and wt% Dex payload (90% porosity and 2.9 wt% Dex). Dex loss during poration can be attributed to increased specimen porosity, the steep concentration gradient imposed on the coatings during salt-leaching/gas-foaming fabrication steps, and heat-induced enhanced drug and polymer chain mobility. [59].
Differential Scanning Calorimetry showed that there was no evidence of crystallized Dex present in porous coatings (Figure 9), indicating that Dex was molecularly dissolved within the polyurethane matrix.

Dex-loaded porous coatings exhibited a fast initial release followed by a slower secondary release characteristic of monolithic drug delivery from a polymer matrix (Figure 10). Daily Dex release from coatings over the 15-day release period was within the therapeutic rage for the treatment of localized acute inflammation (Table 4). Interestingly, changes in coating porosity (surface area) did not significantly affect drug release rates. Therefore, the high drug payload of Dex over a short delivery window (2 weeks) cannot be attributed to just simple diffusion of a small molecule drug through a thin and highly porous surface [106, 107]. Tecoflex® 93A is a segmented polyurethane comprised of soft micro-domains of poly(tetramethylene oxide) and hard crystalline micro-domains of bis(4-isocyanatocyclohexyl)methane (H12MDI) and 1,4-butanediol. As a non-polar drug, Dex preferential interacts with the amorphous soft micro-domains of the polyurethane; these regions serve as drug reservoirs while empty hard crystalline micro-domains act as channels for drug release [108, 109]. Similar results were reported by Gupta et al. [106], where the small molecule hydrophobic drug, dapivirine, was released from Tecoflex®
rings for vaginal drug delivery applications. This study also showed that Dex release from the rings was fast and did not follow first-order release kinetics.

*In vitro* sensor responses to glucose challenge studies ([Error! Reference source not found.](#) and Figure 11) showed that sensor response was not hindered by 90% porous coatings. Signal lag and attenuation in low porosity coatings can be attributed to the increased thickness, decreased pore interconnectivity, and reduced total void volume. Functional sensor studies by Koshwanez et al. [35] similarly showed that sensors deployed with highly porous thick coatings (>200 µm) had elevated response times and diminished signal when compared to bare sensors. This effect was accredited to the augmented migration distance required for glucose to reach the sensor surface. Dex release from coatings also did not interfere with sensor functionality (Figure 11b). Studies by other groups utilizing glucose oxidase based sensors did not show signal fluctuations while functioning within the presence of locally released Dex [55, 79, 110]. Based on signal attenuation and lag times, only 90% porous coatings were used for *in vitro* Dex bioactivity and dose response.

Dex’s anti-inflammatory mechanism has been mostly associated with regulation of cytokine production and reduced metabolic activity of immune cells [52]; however, it
has recently been highlighted that Dex may also control inflammatory and repair tissue responses by preferentially inducing apoptosis of active inflammatory cells (monocytes, macrophages, T cells), while protecting against apoptotic signals in cells involved in tissue repair (epithelial cells and fibroblast) [53, 111]. Consequently the apoptotic monocyte assay reported by Schmidt et al. [53] was used to show that Dex released from 90% porous coatings had a dose-dependent therapeutic effect on immune cells in culture, and that this effect was retained after the heat processing during the gas-foaming/salt-leaching step.

In the Schmidt assay the Annexin-V protein preferentially binds to the phosphatidylserine (PS) receptor, which is externalized on the surface during early cell apoptosis. Annexin-V binding therefore can be used as a marker for apoptosis detection on individual cells. Dex released from coatings proved to be capable of inducing apoptosis in a time and dose dependent manner (Figure 12). Both increasing the Dex payload of coatings and increasing the exposure time to Dex increased the number of monocytes positive for Annexin-V. Media samples made from Dex-free porous coatings also did not show enhanced Annexin-V uptake highlighting the role of Dex as an apoptotic regulator of peripheral blood derived human monocytes.
Prior studies have shown that local Dex delivery is able to improve sensor performance past the “break-in period” by decreasing leukocyte migration and inflammation at the implant site [28, 32, 39, 54, 110]. However, when Dex is delivered over the long-term (>1 month) the drug can act as an angiostatic agent leading to reduced microvessel density, vasodilation, and increased vascular permeability at the implant site.

The textured coatings presented delivered the majority of the Dex payload over the initial week in vitro. This release is suitable to take sensors though the break-in period, while the topographical cues presented by the textured surface are intended to activate endothelial cells to start the neovascularization process. Though we demonstrated the in vitro anti-inflammatory effect of coatings, more exhaustive in vivo testing is necessary fully understand their potential as a platform to extend and improve indwelling sensor functionality.

These findings show that textured Dex-releasing coatings have the potential of modulating the early stages of FBR to an indwelling implant. Yet, the integration of Dex-releasing porous coatings into a commercially available glucose sensing platforms would face significant regulatory challenges. As a combination product, the system would require rigorous testing before it can reach the diabetic patient population [11]. Therefore,
industrial enterprises have in-turn resorted to the use of novel algorithms and recalibration schemes to extend the in-vivo functionality of continuous glucose sensors [12, 16].

Glucose sensing systems have on occasion reported measurements up-to several months in-vivo [16]; however, glucose readings from these studies are still overly numerically and clinically inaccurate to pass regulatory approval and cannot be used as the sole means for programed insulin delivery [17, 18]. Therefore, the implementation of biomaterial and drug delivery strategies to extend sensor function should continue to be explored. Approaches such as localized Dex-release and delivery of topographical cues from the sensor surface could drive the generation of a desirable tissue micro-environment that could result in extended in-vivo sensor functionality.

4.6 Conclusions:

Highly porous Dex-loaded coatings were fabricated by the gas-foaming/salt-leaching technique. Coatings had a controlled pore size and interconnected microstructure. A therapeutic level Dex was loaded into coatings and proved to be in a molecularly dissolved state. Dex release from coatings showed a typical initial fast release followed by a steady release with a high dependency in drug loading over a 15-day
period. Porosity did not affect overall Dex release kinetics, however decreasing coating porosity increased sensor signal lag-time and attenuation. Therefore, 90% porous Dex-releasing coatings were determined to be best for bioactivity testing. Dex released from coatings was able to induced apoptosis of human derived peripheral blood monocytes in a time and dose dependent manner. Future work should focus on in vivo testing of these coatings, which will allow us to fully assess if the combinatorial strategy of anti-inflammatory drug release and delivery of topographical cues from coatings could encourage tissue in-growth and angiogenesis while reducing the immune response to implanted sensor leads.
5. Chapter 5: Specific Aim 2 - Characterization of the foreign body response to porous, dexamethasone-releasing coatings in healthy and diabetic animal models

5.1. Synopsis:

Continuous glucose sensor technologies have seen limited adoption by patients due to untimely failure caused by undesirable tissue-sensor interactions. This chapter presents the in vivo characterization of porous Dex-releasing polyurethane coatings designed to attenuate local inflammation and promote vascularization in the tissue-sensor interface. Initially, acute animal studies were used to determine the appropriate Dex payload for the implanted porous coatings. A more comprehensive study was then performed by deploying porous polyurethane coatings on fully subcutaneous sensor-surrogate implants in both healthy and diabetic rat animal models. In healthy subjects, histological observations and quantification showed that Dex-releasing porous coatings were capable of diminishing inflammation during early time points while promoting the formation of vascularized network of the tissue-sensor interface. Also, differences in wound healing patterns between healthy and diabetic subjects were observed; diabetic rats showed lower levels of inflammation and vascularization of the tissue surrounding implants when compared to their normoglycemic counterparts. These results suggest that
deploying Dex-releasing porous coatings on the surface of sensors might be a promising strategy to remediate the biocompatibility-driven failure of implantable glucose sensors.

5.2. Introduction:

Once implanted, sensors for continuous glucose monitoring are presented with a harsh and dynamic microenvironment. This changing environment makes it difficult to access the effects of such Dex-loaded porous coatings on the FBR utilizing in vitro assays and systems. Therefore, an in vivo evaluation is necessary to study how porous Dex-releasing porous coatings affect the tissue response to an indwelling implant.

While the ultimate goal of this research is to extend the functional life of glucose sensors, testing of coatings directly on percutaneous leads may introduce deleterious variables such as micro-motion, infection, sensor extrusion that will prevent the evaluation of the effects of coatings on the FBR. Therefore, a fully subcutaneous Tygon® tubing implants were used in place of percutaneous sensors for this study.

Evaluation of our coatings in a diabetic model will be the most relevant from a clinical perspective. Altered wound healing and a higher susceptibility to infection are among the most common complications experienced by diabetic patients. In order to better assess the poorly understood wound-healing response to glucose sensor
implantation in diabetic subjects, coatings were also implanted in the dorsum of a chemically induced diabetic rat animal model.

In this study, we present the *in vivo* characterization of Dex-releasing porous polyurethane coatings for needle type glucose sensors. We first performed a study to determine the maximum dosage of Dex that could be delivered from our coating without having systemic effects. After determining the optimal Dex dose, the effects of coatings on the FBR response was evaluated in healthy and diabetic animal models. We used Tygon® tubing implants of similar dimensions and mechanical properties as surrogates for implanted sensors. In order access the effects of coatings on the early stages of the FBR, we performed H&E stains and a macrophage specific stains. Moreover, we quantified the macrophage density in a 100 µm zone around implants. We also assessed the effects of coatings on the later stages of the FBR response by performing Masson’s thichrome stain to determine collagen density and a CD31 antibody stain to assess vessel formation. The percent of collagen and vascular density around implants was also assessed.
5.3. Materials and Methods:

5.3.1. Fabrication of Dex-Releasing Porous Coatings

As detailed in section 4.3.1, porous coatings were fabricated using the gas-foaming/salt-leaching technique. Briefly, a 6.5 wt % solution of polyurethane was prepared by dissolving Tecoflex® 93A pellets (Lubrizol, Technologies) in a solution of 25:75 ethanol to chloroform ratio. Pharmaceutical grade Dexamethasone (Sigma, D9184) was dissolved in the polymer solution and stirred until clear. Sieved (50-75μm) ammonium bicarbonate salt particles (MP Biomedicals, 150107) were added to the polymer solution and homogeneously mixed. Table 6 lists the amount of Dex and ammonium bicarbonate porogen added to polyurethane stock solutions to produce various specimen compositions. Dex-free porous coatings were fabricated by not adding Dex to the polymer solution.

Table 6. Porogen and Dex added to stock polymer solution to produce coatings different porosities and Dex loadings

<table>
<thead>
<tr>
<th>Intended % Porosity</th>
<th>Porogen (g)</th>
<th>Intended wt % Dex</th>
<th>Dex (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>1.5</td>
<td>2.9</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7</td>
<td>45</td>
</tr>
</tbody>
</table>
Polymer films were dip-coated onto copper wire mandrels (Belden, 20 AWG) and allowed to dry for 1 h. Films were porated by placing the polymer coated mandrels into DI water for 5 min at 90 °C to allow gas-foaming/salt-leaching to occur. Porated films were then quickly quenched in 4 °C deionized water for 20 min. Films were allowed to dry in overnight in a desiccator, and cut to a length of 1.5 cm in order to fit over the sensing tips of Medtronic MiniMed SOF-SENSOR™ sensors.

5.3.2. Preparation of Implants

Medical Grade Tygon® tubing (formulation S-54-HL Saint-Gobain, Courbevoie, France) was selected for testing of the foreign body response. Tygon® implants were of similar dimensions (1.5 cm long and 0.8 cm outer diameter) and mechanical properties to the tips of Medtronic MiniMed SOF-SENSOR™. As shown in Figure 13, bare, Dex-free and Dex-loaded porous coatings, and were slid onto Tygon® implants. The coatings fitted snugly over the Tygon® tubing and did not need further affixing. Bare and coated implants were ethylene oxide sterilized and allowed to out-gas for at least 7 days prior to implantation. Bare Tygon® tubing was used as a positive control because Tygon® is a silicone known to undergo fibrous encapsulation when subcutaneously implanted [112].
5.3.3. Implantation Procedure

All National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication no. 85-23 Rev. 1985) were observed. Approval for these studies was granted by the Institutional University Animal Care and Use Committee at Duke University prior to initiation of these studies. Sprague-Dawley rats (200-300 g; CD-type, Charles River Labs, Raleigh, NC) were shaved and prep prior to surgery. After anesthesia induction with 3% isoflurane in oxygen, the dorsal areas were shaved and the skin prepped with chlorhexidine and alcohol three times. The rats were placed in the prone position and a sterile field was created over the dorsum. All implants were inserted via a
trocar introducer. A sterile 12-gauge needle was used to access the subcutaneous plane; implants were inserted into the needle and advanced into the rat subcutis using a plunger. The needle and plunger were simultaneously drawn back while the implant stayed in the desired site. The minimal cutaneous wound did not require primary closure
Figure 14. Implantation procedure of Tygon® implants that were either bare, coated with Dex-free porous coatings or Dex-releasing porous coatings. Black box highlights 12 gauge needle and implant. Red boxes highlight the minimal cutaneous wound and the implant location within the subcutaneous space upon explantation.
5.3.4. Dexamethasone Systemic Effects Study in Healthy Rats

A pilot study was conducted in order to assess the systemic *in vivo* effects of coated implants loaded with Dex. Porous coatings that were Dex-free or contained 0.7, 1.4, and 2.9 wt% Dex were implanted in the dorsal subcutis of rats (n = 2 for each treatment). Uncoated Tygon® tubing was also implanted. All samples were explanted 14 days post-implantation. Systemic Dex effects were monitored by noting the rat's weight and overall-health every 3-days for the duration of the study, and by evaluating the tissue response to uncoated Tygon®.

5.3.5. In-vivo Evaluation of Dexamethasone-Releasing Coatings in Healthy Rats

Samples of 90% porous coatings with 0.7 wt% Dex, which did not exhibit system effects, were implanted in the dorsal subcutis of rats along with Dex-free 90% porous coatings and bare Tygon® controls. Samples were explanted at 3, 7, 14, and 21 days post-implantation (n=28). The Tygon® tubing implants were randomly rotated through the positions outlined in Figure 15 to minimize positional artifacts.
5.3.6. Generation, Monitoring and Implantation of Diabetic Rat Animal Model

Diabetes was induced Sprague-Dawley Rats (200-300 g) using multiple intraperitoneal injections of STZ. Prior to STZ treatment rats will be fasted for 8 hrs. A tail prick was performed to measure initial fasting blood glucose and rat weight was also recorded. Daily i.p. injections of STZ (40 mg/Kg) were performed for 3 consecutive days. Five days after initial STZ treatment rats were again fasted for 3 hrs and blood glucose was recorded. Diabetes onset was considered when fasting blood glucose readings were > 350 mg/dL. In the event that fasting blood glucose was < 350 mg/dL an additional STZ injection (40mg/dL) was administered. This procedure was repeated every 2 days until
fasting blood glucose > 350 mg/dL. In order to assure that diabetic side effects such as altered wound healing and altered immune response have taken hold, implantation of coatings was performed 2-weeks after diabetic on-set. Implantation of coatings followed the protocol outlined in section 5.3.3, and the retrieval schedule as described in section 5.3.5. (n=28; 7 rats per time point).

5.3.7. Evaluation Histological Samples

Tissue samples from pilot and in vivo subcutaneous implant studies in healthy and diabetic rats were flash frozen using liquid nitrogen immediately upon explanation and kept at -80 °C. Serial cryosections of 10 µm thickness were cut. Slides were kept at -80 °C until staining and mounting was performed.

5.3.8. Hematoxylin & Eosin (H&E) Staining

H&E stain was used to qualitatively evaluate the tissue response to implants. H&E 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 then submerged in Mayer’s Hematoxylin medium for 2 minutes and rinsed in tap water until water became clear (~5 min). Slides were placed in an Eosin Y stain for 45 seconds, and then dehydrated through a series of 75 - 100%
ethanol solutions. Slides were then placed in ethanol for 5 minutes. Slides were transferred to Citrisolv medium 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. Slides were mounted and stored in a dry environment until imaging.

5.3.9. Masson’s Trichrome Staining

Masson’s trichrome stain was used to assess collagen deposition around implants. Masson’s trichrome kit was purchased from Sigma-Aldrich (St. Louis, MO). Frozen slides were first placed in Bouin’s solution overnight at 4 °C. Slides were then removed from Bouin’s solution, rinsed with D.I. water, and placed to dry under a fume 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 for 1 minute and rinsed with DI water. Slides were then transferred to a Phosphomolybdic and Phosphotungstic Acid Solution for 5 minutes, followed immediately by 10% Aniline Blue solution for 1 minute. The slides were rinsed three times in fresh solutions of 1% acetic acid and allowing them to sit for 2 min. After rinsing in acetic acid, the slides were then dehydrated consecutively through a series of
75%-100% ethanol solutions. Slides were rinsed and placed in Citrisolv for 2 minutes. Slides were then dipped in 100% ethanol and allowed to dry for 1-2 minutes before mounting. Slides were mounted and stored in a dry environment until imaging.

5.3.10. CD31 Immunochemical Staining

Donkey serum and secondary antibody (donkey anti-mouse coupled with DyLight-488) were purchased from Jackson Immunoresearch (West Grove, PA). Primary antibody (mouse monoclonal anti-Rat CD31) was purchased from BD Biosciences (San Jose, CA). Hoechst 33342 was purchased from Sigma- Aldrich (St. Louis, MO). FluorSave™ Reagent was purchased from EMD Millipore (Darmstadt, Germany). Frozen slides were first fixed in acetone for 20 minutes in @ 4 °C. Slides were then removed from acetone and allowed to dry under a fume hood for 30 min @ RT. A liquid blocking pen was used to encircle tissue samples. After 1 hour, the samples were washed 1x with 1x PBS (-/-) for 5 minutes. Slides were blocked for 30 minutes at RT in a blocking solution of 5% donkey serum. The buffer was then replaced with the primary antibody (diluted 1:200 in blocking solution) for 1 hour. Tissue samples were rinsed 3 times for 5 minutes with 1x PBS (-/-). Secondary antibody solution (diluted 1:100 in blocking solution) was then applied to the tissue samples and allowed to incubate for 30 minutes in the dark. The
slides were then rinsed with 1x PBS (-/-) in the dark for 5 minutes. Hoechst solution (1 mg Hoechst 33342 in 4 ml DI water, diluted 1:100 in 1x PBS (-/-)) was applied to the samples for 5 minutes in the dark. The slides were again washed for 5 minutes with 1x PBS (-/-) in the dark. Slides were mounted using FluorSave™ Reagent, and stored protected from light at 4 °C until imaging.

5.3.11. CD68 Immunochemical Staining

Donkey serum and secondary antibody (donkey anti-mouse coupled with DyLight-488) were purchased from Jackson Immunoresearch (West Grove, PA). Primary antibody (mouse monoclonal anti-rat CD68, MCA341R) was purchased from AbD Serotec (Raleigh, NC). 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 TBS (-/-) for 5 minutes. Slides were blocked for 1 hour at RT in a blocking solution of 5% donkey serum. The buffer was then replaced with the primary antibody (diluted 1:100 in blocking solution) for 1 hour. Tissue samples were rinsed 3 times for 5 minutes with 1x PBS (-/-). Secondary antibody solution (diluted 1:100 in
blocking solution) was then applied to the tissue samples and allowed to incubate for 1 hour in the dark. The slides were then rinsed with 1x TBS (-/-) in the dark for 5 minutes. Hoechst solution (1 mg Hoechst 33342 in 4 ml DI water, diluted 1:100 in 1x PBS (-/-)) was applied to the samples for 5 minutes. The slides were again washed for 5 minutes with 1x TBS (-/-) in the dark. Slides were mounted using FluorSave™ Reagent, and stored protected from light at 4 °C until imaging.

5.3.12. Histological Image Acquisition and Analysis

Images of H&E and Masson’s trichrome stained samples were collected using the 10x objective on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS-2Mv Digital Camera (Nikon Inc., Melville, New York). Images were taken by stitching individual 20x images to generate 6x6 large-image fields. Images of CD31 and CD68 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). Images were taken by stitching individual 20x images to generate 6x10 large-image fields. FITC filter was used to obtain the fluorescent images.
5.3.13. Collagen Capsule Density

As shown in Figure 16A, the density of the collagen in the capsule that formed around Tygon® tubing implants was calculated by averaging four 50 x 50 μm² fields per tissue sample using a previously developed MATLAB (The MathWorks, Natick, MA) program. This program determines the percent collagen from images of Masson’s Trichrome stained tissue sections [35]. In short, 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. This ratio is then multiplied by 100 to obtain percent collagen for that field.

5.3.14. Microvessel and Macrophage Density

As shown in Figure 16B & C, the number of microvessels around implants was determined from CD31 stained sections, while the number of macrophages was determined from CD68 stained sections. For each image, a “ring-like” 100 μm wide zone beginning from the edge of the implant and moving outward was defined. Using ImageJ (National Institutes of Health, Bethesda, MD) this zone was selected and overlaid onto thresholded CD31 or CD68 images. Using the ‘analyze particles’ function the number of structures (count number) and the size of the selected zone in μm² were obtained. These
measurements were then used to calculate the number of microvessels (CD31) or macrophages (CD68) per mm$^2$.

**5.3.15. Statistical Analysis**

All data is presented as means ± standard error of the mean. Data were tested for normality via Shapiro-Wilk tests, and quantile-quantile plots. Because the data were not normally-distributed, they were analyzed using the Wilcoxon Each Pair test. The Bonferroni procedure was applied to control the familywise error rate and to determine significance. Statistical significance was determined as $p \leq 0.05$. 
Figure 16. Photomicrographs of implants coated with Dex-loaded porous coatings after 21 days in rats. a) Masson’s trichrome images were used to compute the collagen density of the foreign body capsule. Four random 50x50 µm² fields (red squares) were analyzed per image. CD68 (b) and CD31(c) images were used to calculate the density of macrophages and microvessels respectively around implants. For each image, a “ring-like” 100 µm wide zone beginning from the edge of the implant (white dotter line) and moving outward (red dotted line) was defined and analyzed. Scale bars = 200 µm for (a) and 400 µm for (b) & (c).
5.4. Results:

5.4.1. Dexamethasone Systemic Effects Optimization in healthy rats

After 14 days, rats implanted with 2.9 wt% Dex porous coatings exhibited 16% to 11% weight decrease as well as atrophy and thinning of the dermal tissue; whereas rats implanted with 1.4 wt% Dex porous coatings did not exhibit external signs of adverse Dex effects. However, histological examination of bare positive control implants showed an impaired inflammatory response when compared to controls from rats implanted with Dex-free coatings (data not shown). Rats implanted with 0.7 wt% Dex porous were able to mount an appropriate immune response to positive control implants, and showed no external signs of systemic Dex effects. From this study it was determined that 0.7 wt% Dex porous coatings were the most fitting for further in vivo tests in rats.

5.4.2. H&E Evaluation of Subcutaneous Tygon® Tubing Implants in Healthy Rats

To confirm that texturing in combination with Dex release had the desired effect on the surrounding tissue, coatings were deployed on Tygon® tubing of similar dimensions to that of the tip of Medtronic MiniMed SOF-SENSOR™ glucose sensors. Coatings were fully implanted in the rat dorsal subcutis for 3, 7, 14 or 21 days.
Figure 17 shows H&E stained sections of tissue that surrounded 90% porous coatings with 0.7 wt% Dex, and 0 wt%, and bare Tygon® positive control for 3, 7, 14 and 21 days after initial implantation. In general, the Dex loaded coatings showed decreased immune cell infiltration to the implant site when compared to Dex-free porous coatings and the bare controls. Moreover, the acute inflammatory response to Dex-free porous coatings was comparable in magnitude to that of the positive Tygon® controls.

The H&E images show that the implant sites with Dex-free porous and bare Tygon® controls displayed a very strong inflammatory response with polymorphonuclear leukocytes, monocytes and macrophages surrounding the implants at days 3, 7 and 14. During the same time frames, Dex-releasing coatings showed noticeable attenuated response with little edema and a small numbers of immune cells actively surrounding the implant. Dex-releasing coatings had less immune cells surrounding the implants than the bare or Dex-free coatings. However, the effect of Dex treatment appeared to stop after 14 days; by day 21 the intensity of the inflammatory response around implants of various treatments was no different.
Figure 17. H&E stained images of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in Healthy Rats. Bare and Dex-free implants show high levels of inflammation at days 3, 7. Tygon® implants deployed with Dex-releasing porous coatings show decreased inflammation at days 3 and 7. By day 21, a thin ring of cells was visible on the surface of Dex-releasing porous coatings. Error bars = 200 µm.
5.4.3. CD31 Evaluation of Subcutaneous Tygon® Tubing Implants in Healthy Rats

The angiogenic potential of porous coatings was monitored by measuring CD31 expression around implants. CD31 (PECAM-1) is a transmembrane glycoprotein that is highly expressed in endothelium. Its localization at the endothelial cell junctions suggests an important role in trans-endothelial cellular migration [113]. Therefore, preferential localization of CD31 can be used to determine neovessel formation and angiogenesis. As presented in Figure 18, bare implants did not experience an increase in blood vessel formation in direct apposition to the implant by day 14 or 21. Implant sites with Dex-free porous coatings experienced an increase in CD31 starting at day 14 which was maintained through the 21 days. In contrast, Dex-loaded porous coatings showed increased vascularization only at day 21 post-implantation. Finally, there was no difference in the angiogenic potential of the implants at days 3 and 7 post-implantation. Also, it was observed that some vessels formed in the space between the sensor and the porous coatings however, this effect was random and found in a small subset of samples.
Figure 18. CD31 photomicrographs of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in Healthy Rats. No difference in vascularity between implants was observed for days 3 and 7. Dex-free porous coated Tygon® implants showed increased vascularity by day 14. Both Dex-free and Dex-Releasing porous coated Tygon® implants showed increased vascularity by Day 21. Error bars = 200 µm.
5.4.4. Collagen Density of Tissue Surrounding Tygon® Tubing Implants in Healthy Rats

Figure 19 shows the Masson’s trichrome stained images of bare, Dex-free porous and Dex-loaded porous coated Tygon® implants after 3, 7, and 21 days post-implantation. By day 21, bare implants show the formation of an organized collagen fibrils in direct apposition to Tygon® implants. Porous coatings show the formation of loose collagenous tissue that was well integrated into the surrounding subcutaneous tissue. Dex-releasing porous implants had similar response that of Dex-free porous coatings showing disruption of collagen fibril formation by day 21.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>% Area of Collagen (%) by treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
</tr>
<tr>
<td>Day 3</td>
<td>15.0 ± 12.3</td>
</tr>
<tr>
<td>Day 7</td>
<td>16.5 ± 13.5</td>
</tr>
<tr>
<td>Day 14</td>
<td>22.9 ± 10.9</td>
</tr>
<tr>
<td>Day 21</td>
<td>35.8 ± 13.6</td>
</tr>
</tbody>
</table>

As outlined in Table 7, there was no difference in collagen density between subcutaneous implants at 3 and 7 days post-implantation. By day 14, the collagen density
of the tissue around bare implants increased from 16.5 ± 13.5 to 22.9 ± 10.9% while the collagen density around porous coatings regardless of Dex-loading increased by ~2%. However, by day 21 the mean collagen density around porous coated sensors regardless of Dex-loading was about 1-fold lower than that of bare sensors (about 18% collagen for porous coatings and 35.8% for bare sensors).
Figure 19. Masson’s Trichrome images of Tygon® implants and subcutaneous tissue after 3, 7, 14, and 21 days in Healthy Rats. By day 21, the formation of organized collagen capsule was observed on bare sensor implants. Porous implants regardless of Dex-loading were able to disrupt the formation of this capsule. Error bars = 200 µm.
5.4.5. Macrophage and Vessel Density of Tissue Surrounding Tygon® Tubing Implants in Healthy Rats

Figure 20 displays the density of macrophages within the 100 µm zone surrounding Tygon® tubing implants. Dex-releasing implants significantly showed the lowest density of Macrophages at days 3, 7, and 14, with densities of $28 \pm 4, 38 \pm 5, 43 \pm 2$ cells/µm² respectively (Figure 20). However, by day 21 there was not difference in the density of macrophages regardless of implant type.

Figure 21 shows the density of blood vessels in the same 100 µm region. There was no difference in the density of blood vessels surrounding implants at days 3 and 7, with a vessel density under 50 vessels/µm² for all implants regardless of treatment. A significant increase in vessel density was observed in Dex-free porous coatings starting at day 14 with a blood vessel density of $66 \pm 8$ vessels/mm² with respect to bare Tygon® tubing and Dex-free porous coatings. By day 21, both Dex-free and Dex-loaded porous coatings had significantly higher vessel density when compared to non-coated implants. Dex-loaded implants were able to attenuate the inflammatory response, while porous coatings regardless of Dex-loading were increased vascularity around implants.
Figure 20. Density of macrophages within the 100 µm zone surrounding Tygon® implants in Healthy Rats. The tissues surrounding Dex-free implants were significantly inflamed when compared to the tissue surrounding the implants with Dex releasing coatings at days 3, 7 and 14. However, by day 21 there was no difference in the number of inflammatory cells surrounding the implants.
Figure 21. Density blood vessels within the 100 µm zone surrounding Tygon® implants in Healthy Rats. The tissues surrounding implants showed no differences in vascularity at days 3 and 7. On day 14, vascularity around Dex-free porous implants significantly increased. By day 21, vascularity for all porous implants regardless of Dex-loading had significantly increased when compared to controls.
5.4.6. H&E Evaluation of Subcutaneous Tygon® Tubing Implants in Diabetic Rats

Figure 22 shows tissue sections taken from the implant sites of 90% porous coatings with 0.7 wt% Dex, and 0 wt% Dex, and bare Tygon® positive control for 3, 7, 14 and 21 days after initial implantation in diabetic rats. Images show that Dex-free porous coatings and bare Tygon® controls resulted in a mild inflammatory response characterized by the presence of some neutrophils and lymphocytes accumulated at implantation site. In contrast, the tissue surrounding Dex-loaded coatings showed no signs of inflammation.

Though both healthy and diabetic rats experienced an acute inflammatory response to implants, the intensity of the response in hyperglycemic subjects is markedly decreased in comparison to healthy rats (Figure 17). The intensity of the inflammatory cell infiltration to the bare and porous Dex-free implant sites on days 3 and 7 in diabetic rats is much less than healthy rats.
Figure 22. H&E stained images of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in diabetic rats. Error bars = 200 µm.
5.4.7. CD31 Evaluation of Subcutaneous Tygon® Tubing Implants in Diabetic Rats.

Figure 23 shows CD31 photomicrographs of Tygon® implants and subcutaneous tissue at 3, 7, 14 and 21 days post-implantation in diabetic rats. Bare, Dex-free and Dex-loaded porous coatings did not experience an increase in blood vessel formation adjacent to the implant at any of the experimental time points. Also, it was observed that some vessels formed in the space between the sensor and the porous coatings however, this effect was random and found in a small subset of samples.

An approximately 100 µm thick vessel-free zone was observed around all implants regardless of treatment throughout the length of the study. When compared to normoglycemic tissues, diabetic tissues surrounding the Dex-free porous coated implants did not experience the same increase in CD31 expression at days 14 and 21. Moreover, Dex-loaded porous coatings did not show the same levels of vascularization at day 21 post-implantation that were observed when the coatings were implanted in healthy subjects. No differences in the level of vascularization of the tissues surrounding implants was observed during any of the experimental time-points.
Figure 23. CD31 photomicrographs of Tygon® implants and subcutaneous tissue after 3, 7, 14 and 21 days in diabetic rats. Error bars = 200 µm.
5.4.8. Collagen Density of Tissue Surrounding Tygon® Tubing Implants in Diabetic Rats

Figure 24 shows the Masson’s trichrome stained images of bare, Dex-free porous and Dex-loaded porous coated Tygon® implants after 3, 7, and 21 days post-implantation in diabetic rats. No difference in the density of collagen was found between implants during any of the time points. When compared to normoglycemic tissues, tissue surrounding bare implants in diabetic rats did not show the formation of an organized collagen fibrils by day 21, but rather experienced the formation of loose collagenous tissue. This same loose collagenous tissue was observed around Dex-free and Dex-loaded porous coatings.

Table 8. Collagen density in tissue adjacent to Tygon® implants in diabetic rats (n=7)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>% Area of Collagen (%) by treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
</tr>
<tr>
<td>Day 3</td>
<td>16.1 ± 8.3</td>
</tr>
<tr>
<td>Day 7</td>
<td>16.4 ± 9.3</td>
</tr>
<tr>
<td>Day 14</td>
<td>17.2 ± 11.2</td>
</tr>
<tr>
<td>Day 21</td>
<td>18.3 ± 10.1</td>
</tr>
</tbody>
</table>
As outlined in Table 8, there was no difference in collagen density between subcutaneous implants at days 3, 7, 14, and 21 post-implantation. In contrast with healthy subjects the collagen density around bare Tygon® tubing implants in diabetics did not double from day 3 to day 21, but rather stayed ~ 16-17%. Moreover, lower collagen densities were not observed in the tissue surrounding Dex-free and Dex-loaded porous coatings when compared to bare implants at day 21.
Figure 24. Masson’s trichrome images of Tygon® implants and subcutaneous tissue after 3, 7, 14, and 21 days in diabetic rats. Error bars = 200 µm.
5.4.9. Macrophage and Vessel Density of Tissue Surrounding Tygon®
Tubing Implants in Diabetic Rats

Figure 25 displays the density of macrophages within the 100 µm zone
surrounding Tygon® tubing implants in diabetic subjects. Macrophage density increased
with time for all implants. Interestingly, at day 3 bare implants diabetic rats showed lower
macrophage densities of 28 ± 15 when compared to the same implants in healthy rats 67 ±
9 (Figure 20). Moreover, Dex-releasing implants showed the lowest density of
macrophages at days 3, 7, and 14, with densities of 20 ± 6, 31 ± 9, 47 ± 22 cells/ µm²
respectively. However, this differences were not statistically significant.

Figure 26 shows the density of blood vessels within the 100 µm zone surrounding
Tygon® tubing implants in diabetic subjects. There was no significant differences in the
vessel density of the tissue surrounding implants. All implants regardless of treatment
showed a vessels density around 10 – 20 vessels/mm² within the 100 µm zone. Moreover,
vessel density around Dex-free and Dex-loaded porous coatings did not increase with
time regardless of treatment. This is in contrast, to the increased observed in healthy rats.
When compared to results in healthy rats, vessel density around porous coated implants
was lower in diabetic subjects than in healthy rats at days 14 and 21.
Figure 25. Density of macrophages within the 100 µm zone surrounding Tygon® implants in Diabetic Rats. Macrophage density increased with time for all implants regardless of treatment. Tissue surrounding Dex-releasing coatings had the lowest macrophage densities for all time points. Macrophage densities were on average lower in diabetic tissues when compared to healthy rats.
Figure 26. Density blood vessels within the 100 µm zone surrounding Tygon® implants in Diabetic Rats. The tissues surrounding implants showed no differences in vascularity at any time points. On day 14 and 21, vascularity around Dex-free porous implants was lower when compared to healthy rat tissues. By day 21, vascularity for all porous implants regardless of Dex-loading was lower when compared to healthy rat controls.
5.5. Discussion:

The commercial success of implantable continuous glucose monitoring technologies has been hindered by the premature failure of such devices \textit{in vivo} \cite{7, 10, 24, 37}. This failure has been attributed to the FBR and the formation of a fibrous capsule that prevents the appropriate transport of analyte to the glucose sensor surface. Most recently, \textit{in vitro} and modeling studies have identified the consumption of glucose by inflammatory cells and lack of vascularity around implanted sensor lead as key incidences that lead to premature sensor failure \cite{25, 35, 114}. Therefore, a system that curbs inflammation while promoting the thorough vascularization of the tissue surrounding of implanted sensor leads is desirable.

In Chapter 4, we reported on the characterization and \textit{in vitro} testing of Dex-releasing porous polyurethane coatings to improve the performance of glucose sensors \cite{115}. This system aimed at improving sensor function by combining angiogenic texturing with localized and timed release of a powerful anti-inflammatory agent (Dex). We demonstrated that Dex-releasing porous coatings fit snugly on the surface of Medtronic MiniMed SOF-SENSOR\textsuperscript{TM} glucose sensors, had a controlled porous microstructure, minimally interfered with sensor performance \textit{in vitro}, and fully released bioactive
amounts of Dex for 14 days. However, questions regarding the effects of coatings on the pathology of the FBR still remained.

Firstly, a pilot animal studies were performed to determine whether porous coatings with payloads of 0.7, 1.4 and 2.9 wt% Dex would elicit an anti-inflammatory repose in rodents without any apparent system effects. Not surprisingly, highly porous coatings with 0.7 wt% Dex loading were the best suited for in vivo deployment since they were able to suppress inflammation locally, while still allowing for adequate immune function at distant sites. This formulation initially delivers ~ 30ug/day of Dex (Table 4) which is within the range of the recommended effective Dex daily dosage [55].

Coatings were further tested in a more through animal study to determine Dex’s effects on the wound healing. Tygon® tubing was chosen for implantation instead of non-functional glucose sensors because hydrogen peroxide builds up in the subcutaneous space adjacent to the glucose oxidase present on non-functional sensors. In fully functional sensors, the applied voltage quickly breaks down the hydrogen peroxide generated by glucose oxidase before it can accumulate.

In our study, implant sites with Dex-free porous coatings and bare Tygon® controls showed a strong inflammatory responses with dense fields of
polymorphonuclear leukocytes, monocytes and macrophages surrounding the implants during early Tim points days 3, 7, and 14. In contrast, Dex-loaded coatings showed a significantly attenuated inflammatory response with only a few macrophages surrounding the implant. These results demonstrate that Dex released from coatings is not only bioactive but also capable of mediating the immune response to sensor implantation.

Prior studies have shown that local Dex delivery is able to improve sensor performance past the "break-in period" by decreasing leukocyte migration and inflammation at the implant site [28, 32, 39, 54, 110]. However, when Dex is delivered over the long-term (>1 month) the drug can act as an angiostatic agent leading to reduced microvessel density, vasodilation, and increased vascular permeability at the implant site.

It is important to highlight that histological evaluation and in vitro drug release profiles suggest that the anti-inflammatory effects the Dex-releasing coatings take place between days 0 – 14 post-implantation. Figure 20, shows that by day 21 there was no difference in the number of macrophages surrounding coatings suggesting that the Dex-load from the coatings had been mostly released by that time point.
Due to the timed release of Dex and the presentation of texturing cues, Dex-releasing porous coatings promoted the enhanced vascularization of tissues. Dex has been known to act as angiostatic agent and hinder endothelial cell migration to the wound site [32, 60]. Therefore it is important for Dex-releasing coatings to fully deploy their Dex load, to allow for the formation of capillaries. When assessing the vascularization around coatings, we observed that by day 21 both the tissue surrounding Dex-free and Dex-releasing porous coatings showed increased vascularization in healthy rats (Figure 21).

By day 21, Dex concentration at the wound site is minimal and topographical cues from coatings take over as the main mediators of the tissue response. Consequently, increased vascularization (CD31 expression) was observed at the implant site. Several studies have reported on the angiogenic effects of texturing to increase vascularity around medical implants [18, 35, 63, 76, 115]. Though cellular pathways through which topographical cues promote angiogenesis and tissue integration, it is believed that pores within the 5-100 µm provide an optimal environment for transport of analytes and migration of endothelial cells [75].

Moreover, the differential levels of vascularization between Dex-free and Dex-releasing coatings at day 14 can also be attributed to Dex release kinetics from porous
coatings. At 14 days, Dex is still being released from porous coatings, thus vascularization at site is diminished.

As expected, bare Tygon® tubing implants showed an increase in the collagen density over time, due to the formation of a dense collagen capsule. Collagen density of the tissue surrounding bare implants almost doubled between days 3 and 21. In contrast, collagen density remained between 15-16% around porous coated implants regardless of Dex-loading. Lower collagen density levels around porous coatings can be attributed to both Dex release and texturing effects. Glucocorticoids not only reduce immune cell recruitment but have been linked to the reduction of collagen production by fibroblasts. Dex induced inhibition of collagen production results from a receptor-mediated inhibition of gene expression of type 1 procollagen mRNAs [52, 60, 116]. Moreover, studies comparing collagen density around solid implants to porous implants of (PVA and ePTFE) noted that the density of the capsule that developed around the porous materials was much lower than that of solid materials [18]. Also, Braber et al. has shown that specific features in the size, shape of topographical cues alter the way fibroblasts orient and attach to substrates [117].
These results show that Dex-releasing porous coatings are capable of attenuating inflammation, while disrupting collagen capsule formation and promoting vascularization around implants.

In practice, glucose sensors are mostly prescribed to monitor glucose levels in diabetic patients. Among its many side effects diabetes causes altered wound healing patterns. This altered wound healing patterns, could lead to differences in performance and durability of glucose sensors between healthy and diabetic subjects. Characteristically, diabetic wounds show distorted cytokine production, curbed macrophage and neutrophil function, poorly organized granulation tissue, reduced angiogenic response, and abnormal collagen deposition. Therefore, this study also aimed to characterize the differences between diabetic and healthy wound healing and evaluate the effects of Dex-releasing coatings on the diabetic FBR.

STZ treatment was very effective at inducing hypoglycemia in healthy rats. Out of 28 rats treated with STZ, half became hyperglycemic after 3 shots. The remaining rats needed either one or two additional shots of STZ to achieve a fasting hypoglycemic state. After hypoglycemic on set, weekly blood glucose levels remained above 350 mg/dL for the duration of the study. STZ leads to uncontrolled glucose levels by selectively binding
to the transport protein GLUT2 which is found predominantly in the insulin-producing 
β-cells of the pancreas. Once inside the cell, STZ causes DNA damage leading to β-cell 
death and diminished insulin production [99]. Moreover, wound healing studies have 
shown that genetically healthy rats when made hyperglycemic by STZ administration 
heal less effectively than controls [84].

Though both healthy and diabetic rats experienced an acute inflammatory 
response to implants, the intensity of the response in hyperglycemic subjects is markedly 
decreased in comparison to healthy rats. The intensity of the inflammatory cell infiltration 
at the implant sites on days 3 and 7 in diabetic rats is much less than healthy rats. This 
decreased inflammatory response is caused by the altered immune cell function, cytokine 
production and cell metabolism characteristic of diabetic tissues. It has been reported that 
diabetic patients have a delayed immune response to tissue injury, pathogen or foreign 
body stimuli. This altered cytokine profile leads to deviations in the time and length of 
the inflammatory response and remodeling stages of the FBR.

When implanted in diabetic subjects, porous coatings regardless of Dex-loading 
were not able to promote the formation of a well vascularized tissue around implants by 
day 21. There was no difference between the vascularization of porous coatings and bare
Tygon® controls in diabetic tissues. These results were markedly different from those found in healthy rats, where porous coatings regardless of Dex-loading were capable of significantly increasing vessel density within the 100 μm zone when compared to controls. Differences in responses between diabetic and healthy can be attributed to the reported lower levels or circulating EPCs in diabetic subjects, as well as the impaired homing of these cells to diabetic wound sites, when compared to normoglycemic tissues [95].

In contrast to bare implants in healthy rats, bare Tygon® tubing implants in diabetic tissue did not show a pronounced increase in the collagen density over time. Collagen density of the diabetic tissue surrounding bare implants remained between 16 - 18% while in healthy subjects it increased from 15%-35% from days 3 – 21 post-implantation. These noted differences in collagen deposition between diabetic and healthy tissues is due to the inhibited collagen synthesis by fibroblast found in hyperglycemic tissues [118, 119].

5.6. Conclusions:

Here we demonstrated that controlled and rapid release of Dex in combination with delivery of topographical cues from a coating can successfully mediate FBR to an indwelling implant. Dex release was capable of attenuating initial inflammation and
impair immune cell migration to implant site over a period of 14 days, while
topographical cues were capable of enhancing vascularization around the implant by 21
days. Differences between healthy rats and diabetic subjects were observed. Though
diabetic rats were able to mount an acute inflammatory response, the number of
inflammatory cells surrounding implants was reduced when compared to healthy
controls. Evaluation of the angiogenic potential of porous coatings in diabetic animal
showed that porous coatings regardless of Dex-loading were not able to promote the
formation of a well vascularized tissue around implants. Though these results
demonstrate that coatings were capable of altering and mediating the tissue response to a
foreign implant, it is still necessary to access if the favorable changes in tissue
microenvironment lead to improved sensor function *in vivo*. Therefore, testing of coating
on a functional sensor platform is required.

6.1. Synopsis:

Based on the results from Specific Aim 2, it was established that Dex-releasing coatings were 1) capable of attenuating inflammation in within the initial 14 days post-implantation and 2) able to promote vascularization of the sensor tissue interface. However, the effect of this changes in tissue micro-environment on glucose sensor function still need to be elucidated.

In order to evaluate the effects of the tissue microenvironment created by Dex-releasing porous coatings on sensor performance, we deployed coatings from the tips of percutaneous Medtronic MiniMed™ SOF-Sensor glucose sensors. This sensors were subsequently implanted in the dorsum of diabetic and healthy rat animal models.

An already optimized animal model was used to implant and secure the sensors to the dorsum of rats. The rat-jacket model was successfully used by Koschwanez et al. [34, 35], where sensor readings for over 21-days in a moving rat were obtained. Moreover, this model was chosen in order to minimize anticipated micro-motion, infection, sensor extrusion issues. To attain three-sensor configuration using the rat-jacket model, slightly
bigger rats (300-350 g) than the ones used in Chapter 5 (250 – 300 g) were used for these studies.

After animal model optimization, 3, 7, and 21 day animal studies were performed to correlate sensor signal strength and response times to changes in immune cell recruitment and vascularization to the tissue surrounding implanted in glucose sensor leads. These time points were select based on results presented in Chapter 5. The anti-inflammatory action of Dex appeared to be the greatest at 3 days post-implantation while the angiogenic activity of the porous coatings was highest after 3 weeks in healthy rats.

To better characterize sensor response to a drastic glucose change, implanted sensors were subjected to functional challenges through periodic glucose boluses. Sensor signal, sensitivity and attenuation were recorded and analyzed. The tissue sensor interface was evaluated by performing histological measurements and immunohistochemistry. Finally, these studies were also be conducted in a diabetic rat animal models to draw parallels between diabetic and normoglycemic wound healing on glucose sensor function.
6.2. Introduction:

It is a well-established that close regulation of blood glucose levels is the single most important factor in preventing complications from diabetes [120]. Researchers have been developing closed-loop artificial pancreas (AP) systems where real-time changes in glucose used to trigger an insulin pump to the delivery of the appropriate dosages. In recent years, AP development has been stalled due to the unreliability and untimely failure of implantable glucose sensors [3].

Classical continuous glucose sensing systems are amperometric, rely on glucose oxidase for sensing, and are percutaneous in nature [5, 8, 121]. Due to the short in vivo life of this sensors, new fully subcutaneous systems that rely on novel biomaterial platforms, unconventional enzymes, and novel optical technologies for glucose detection are in early stages of development [37, 122, 123]. However, the complexity of development and regulatory approval times of this novel platforms is still of concern. Therefore, it is imperative to also improve upon classical glucose sensing systems for technological advances to reach the public in acceptable timeframes.

Upon implantation a classical glucose sensor is presented with a dynamic microenvironment known as the foreign body response [7, 24]. Within seconds of
implantation, proteins biofoul the sensor surface followed soon by the arrival of immune
cells that attempt to degrade and consume the sensor. Since the sensor cannot be
eliminated by the host, this response typically culminates in the formation of an avascular
fibrous capsule that surrounds the implanted sensor. As a result, blood glucose
measurements from the sensor are often erratic, attenuated, and unreliable [7, 24, 35, 122].

Initial biomaterial strategies to extend glucose sensor functionality focused on
preventing protein adsorption through the incorporation of hydrogel coatings; though
antibiofouling strategies proved to be well suited for acute sensor applications, they
appear to be insufficient to achieve reliable long-term glucose sensor performance [77]
[55]. Most recently, modeling and experimental studies have shown that inflammation
and tissue encapsulation may be the most critical stages affecting sensor function in vivo
[25, 114].

Strategies for the attenuation of inflammation and the promotion of tissue
vascularization have employed the release of “tissue response modifiers”, such as the
local release of growth factors (VEGF, PDGF), nitric oxide [26, 51], or anti-inflammatory
glucocorticoids [39, 54, 55]. Topographical and biomimicry approaches have also been
used to increase vascularization and promote tissue integration around implanted sensor
leads [34, 35, 78, 79]. To date, combined strategies to improve sensor biocompatibility have involved either dual agent release or antifouling gels merged with localized drug delivery.

In Chapters 4 and 5, we reported on the characterization of porous polyurethane coatings with a controllable microstructure, tunable drug loading and bioactive release of Dexamethasone (Dex) [115]. However, questions regarding the effects of coatings on glucose sensor function in vivo still remained. This chapter presents the results of two animal studies that assessed the effect of porous Dex-releasing coatings on Medtronic MiniMed SOF-SENSOR™ glucose sensors implanted in rat dorsal subcutis for up to 21 days. The first study aimed to evaluate if diminished inflammation and increased vascularization of the tissue adjacent to implanted sensor leads to improved sensor function when implanted in healthy rats. The second study, evaluated if there were disparities between healthy and diabetic wound healing patterns, and characterized the effects of Dex-releasing coatings on sensor function when implanted in a diabetic rat animal model.
6.3. Materials and Methods:

6.3.1. Fabrication of Dex-Releasing Porous Coatings

As detailed in section 4.3.1, porous coatings were fabricated by gas-foaming/salt-leaching technique. Briefly, a polyurethane solution of 6.5% w/w was prepared by dissolving Tecoflex® 93A pellets (Lubrizol, Technologies) in a solution of 75:25 chloroform to ethanol ratio. Forty-five milligrams of pharmaceutical grade Dexamethasone (Sigma, D9184) were added to the polymer solution to obtain a desired weight ratio of Dexamethasone/polyurethane solution of 0.7% v/v. The solution was then stirred until clear. One and a half grams of sieved ammonium bicarbonate salt particulates (50-75 μm) were added and homogenously mixed. Concentration of salt particles was varied in ratios to obtain scaffolds of desired porosities. Copper wire mandrels (Belden, 20 AWG) were used in place of glucose sensors to protect glucose oxidase bioactivity. Mandrels were allowed to dry overnight under vacuum. Finally, coatings were cut to a length of 1.5 cm to match Medtronic MiniMed SOF-SENSOR™ tip dimensions.

6.3.2. Preparation of Functional Sensor Implants

Dex-free and Dex-loaded porous coatings were slid onto Tygon® tubing mandrels to prevent deformation during sterilization and handling. Coatings were ethylene oxide
sterilized and allowed to out-gas for at least 7 days prior to implantation. Coating of Medtronic MiniMed SOF-SENSOR™ glucose sensors was performed under sterile conditions in a laminar flow hood. One day prior to surgery, coatings were removed from sterile packaging deployed over the tips of functional Medtronic MiniMed SOF-SENSOR™ glucose sensors (Figure 27). Coatings were further affixed by securing coatings to the external plastic housing of the sensors using USP Class VI medical grade EP42HT epoxy. The epoxy was allowed to cure for 1 hour under the sterile hood. After curing, sensors with and without coatings were placed in sterile packaging and stored in the dark at 4 °C. .
Figure 27. Picture of Medtronic MiniMed glucose Sensor™ with and without highly porous Dex-releasing coatings.

6.3.3. Implantation of Functional Glucose Sensors

All National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication no. 85-23 Rev. 1985) were observed. Approval for these studies was granted by the Institutional University Animal Care and Use Committee at Duke University prior to initiation of these studies. Sprague-Dawley rats (300-350 g; CD-type, Charles River Labs, Raleigh, NC) were shaved and prep prior to surgery. Three Medtronic MiniMed SOF-SENSOR™ glucose sensors were implanted in each healthy or diabetic rat (n=21). These sensors were treated with either a Dex-loaded porous coating, Dex-free
porous coating or have no treatment. Sensors were placed in a circular arrangement on the dorsum of rats (Figure 28) and implanted as described below.

Aseptic technique was followed for all surgical procedures. Briefly, after anesthesia induction, the dorsal areas of the rat were shaved and the skin prepped with chlorhexidine/alcohol 3 times. The rat was then placed in the prone position and a sterile field was created over the dorsum. A 12-gauge needle was used to puncture the dorsal skin. The sensing tip of each sensor with or without coatings was then gently inserted through the needle wound into the subcutaneous plane. The standard external plastic
housing was then secured to the dorsum using braided nylon sutures (4-0). This procedure was repeated to implant the remaining two sensors. Antibiotic ointment was administered around the sutured areas. Implanted sensors were then connected to their respective MiniLink™ external recorder. Sensors and transmitters were further secured to the rat dorsum by wrapping them with Vetrap® wound dressing. The Vetrap® wound dressing was additionally fasten using nylon sutures (4-0). After implantation, one drop of blood was collected from the tail via a needle stick for a blood glucose measurement. The rats were then allowed to recover and returned to their cages where they were housed singly.
Figure 29. Implantation Procedure of Functional Medtronic MiniMed SOF-SENSOR™ Sensors.

Bare, Dex-loaded or Dex-Free porous coated sensor implants.

Outlined sensor placement and created sterile field

Used 12 gauge needle to access the SQ Space

The 12 gauge needle created a tunnel in the subcutaneous space through which sensors were implanted.

Sensors were secured on the dorsum with Vetrap®

Implanted 2+ sensors on the dorsum of rats

Sensors were secured with sutures
6.3.4. *In-vivo* Evaluation of Functional Glucose Sensors in Healthy Rats

Functional Glucose Sensors treated with 90% porous coatings with 0.7 wt% Dex, which did along with Dex-free 90% porous coatings and bare controls. Samples were explanted at 3, 7, and 21 days post-implantation (n=21). The Medtronic MiniMed SOF-SENSOR™ implants were randomly rotated through the positions outlined in Figure 28 to minimize positional artifacts.

6.3.5. Generation, Monitoring and Implantation of Functional Glucose Sensors in Diabetic Rats

Diabetes was induced Sprague-Dawley Rats (250-350 g) using multiple intraperitoneal injections of STZ. Prior to STZ treatment rats were fasted for 8 hrs. A tail prick was performed to measure initial fasting blood glucose and rat weight was also recorded. Daily i.p. injections of STZ (40 mg/Kg) were performed for 3 consecutive days. Five days after initial STZ treatment rats were again fasted for 3 hrs and blood glucose was recorded. Diabetes onset was considered to be when fasting blood glucose readings were > 350 mg/dL. In the event that fasting blood glucose was < 350 mg/dL an additional STZ injection (40mg/dL) was be administered. This procedure was repeated every 2 days until fasting blood glucose was > 350 mg/dL. In order to assure that diabetes induced side effects such as altered wound healing and altered immune response had taken hold,
implantation of sensors was performed 2-weeks after diabetic on-set. Implantation of functional sensors followed the retrieval schedule described in section 6.3.4, the protocol outlined in section 6.3.3. (n=9; 3 rats per time point).

6.3.6. Continuous Glucose Monitoring

Blood glucose concentrations were monitored continuously using the implanted Medtronic MiniMed SOF-SENSORTM glucose sensors until failure of the devices or experiment termination (3, 7 or 21 days post implantation). The glucose sensor continuously monitored glucose levels within each rat. When in contact with interstitial glucose, subcutaneously implanted sensor tips produced an electrical current that was stored in the MiniLinkTM external recorder. The sensor system reported an average current measurement (nA) every 5 minutes that correlated to the blood glucose concentration during that time. Additionally, date and time information for each average current measurement were appended to the data. These average current measurements were downloaded from the MiniLinkTM recorders, utilizing a Medtronic MiniMed iProTM2 Dock and the SMuRF Utility software package. Data was further analyzed using Microsoft Excel and GraphPad Prism software.
6.3.7. Glucose Sampling

Blood glucose levels were monitored every 12-hours post implantation using OneTouch Ultra test strips and blood glucose hand-held system (Milpitas, CA). During each measurement, one drop of blood was collected via a single needle stick to the tail vein. Date and time of each of these measurements was also recorded. This procedure did not require anesthesia since the rats only experienced pain from a single needle stick. These measurements were collected every 12 hours until failure of the implanted continuous glucose sensors or experiment termination (days 3, 7 or 21 post implantation). Glucose sampling data was used to calculate sensor sensitivity values.

6.3.8. Glucose Bolus Tests

Tests to examine the functionality of the sensor were performed at days 1, 3, 7, 14 and 21 post-implantation. Prior to anesthesia induction a tail-vein blood glucose measurement was recorded to establish a glucose baseline. Tail vein blood glucose measurements were performed as described above. After anesthesia induction, each rat was be injected with 5 units/Kg of insulin intraperitoneally. Glucose levels were monitored via tail vein needle sticks and a hand-held meter every 5-10 min until glucose levels are under 100 mg/dL and sustained for 15-20 min. Rats were put under anesthesia.
induction again and a dosage of 2 g/Kg of 50% Dextrose solution was delivered intraperitoneally. Tail vein blood measurements were performed and recorded every 10-15 minutes until baseline glucose levels were reached and sustained for a period of 30 min. In order to test sensor functionality, glucose readings obtained from a handheld meter were compared to current outputs (nA) from continuous glucose sensors. Data from Glucose Bolus test was used to calculate changes in sensor lag and functional disparity between sensors (MARD).

6.3.9. Explantation Procedure

Sensors were recovered at days 3, 7 and 21 post-implantation (n=7 in healthy rats and n=3 in diabetic rats for each time point). Following sacrifice, surgical scissors were used to remove external sutures and separate sensor tips from the external plastic housing. Sensors and surrounding tissue were surgically excised and flash frozen using liquid nitrogen.

6.3.10. Evaluation Histological Samples

Tissue samples from pilot and in vivo subcutaneous implant studies in healthy and diabetic rats were flash frozen using liquid nitrogen immediately upon explanation and
kept at -80 °C. Serial cryosections of 10 µm thickness were cut. Slides were kept at -80 °C until staining and mounting was performed.

6.3.11. Hematoxylin & Eosin (H&E) Staining

H&E stain was used to qualitatively evaluate the tissue response to implants. H&E 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 then submerged in Mayer’s Hematoxylin medium for 2 minutes and rinsed in tap water until water became clear (~5min). Slides were placed in an Eosin Y stain for 45 seconds, and then dehydrated through a series of 75 - 100% ethanol solutions. Slides were then placed in ethanol for 5 minutes. Slides were transferred to Citrisolv medium 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. Slides were mounted and stored in a dry environment until imaging.

6.3.12. Masson’s Trichrome Staining

Masson’s trichrome stain was used to assess collagen deposition around implants. Masson’s trichrome kit was purchased from Sigma-Aldrich (St. Louis, MO). Frozen slides
were first placed in Bouin’s solution overnight at 4 °C. Slides were then removed from Bouin’s solution, rinsed with D.I. water, and placed to dry under a fume 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 for 1 minute and rinsed with DI water. Slides were then transferred to a Phosphomolybdic and Phosphotungstic Acid Solution for 5 minutes, followed immediately by 10% Aniline Blue solution for 1 minute. The slides were rinsed three times in fresh solutions of 1% acetic acid and allowing them to sit for 2 min. After rinsing in acetic acid, the slides were then dehydrated consecutively through a series of 75%-100% ethanol solutions. Slides were rinsed and placed in Citrisolv for 2 minutes. Slides were then dipped in 100% ethanol and allowed to dry for 1-2 minutes before mounting. Slides were mounted and stored in a dry environment until imaging.

6.3.13. CD31 Immunochemical Staining

Donkey serum and secondary antibody (donkey anti-mouse coupled with DyLight-488) were purchased from Jackson Immunoresearch (West Grove, PA). Primary antibody (mouse monoclonal anti-Rat CD31) was purchased from BD Biosciences (San Jose, CA). Hoechst 33342 was purchased from Sigma-Aldrich (St. Louis, MO).
FluorSave™ Reagent was purchased from EMD Millipore (Darmstadt, Germany). Frozen slides were first fixed in acetone for 20 minutes in @ 4 °C. Slides were then removed from acetone and allowed to dry under a fume hood for 30 min @ RT. A liquid blocking pen was used to encircle tissue samples. After 1 hour, the samples were washed 1x with 1x PBS (-/-) for 5 minutes. Slides were blocked for 30 minutes at RT in a blocking solution of 5% donkey serum. The buffer was then replaced with the primary antibody (diluted 1:200 in blocking solution) for 1 hour. Tissue samples were rinsed 3 times for 5 minutes with 1x PBS (-/-). Secondary antibody solution (diluted 1:100 in blocking solution) was then applied to the tissue samples and allowed to incubate for 30 minutes in the dark. The slides were then rinsed with 1x PBS (-/-) in the dark for 5 minutes. Hoechst solution (1 mg Hoechst 33342 in 4 ml DI water, diluted 1:100 in 1x PBS (-/-)) was applied to the samples for 5 minutes in the dark. The slides were again washed for 5 minutes with 1x PBS (-/-) in the dark. Slides were mounted using FluorSave™ Reagent, and stored protected from light at 4 °C until imaging.

6.3.14. CD68 Immunohistochemical Staining

Donkey serum and secondary antibody (donkey anti-mouse coupled with DyLight-488) were purchased from Jackson Immunoresearch (West Grove, PA). Primary
antibody (mouse monoclonal anti-rat CD68, MCA341R) was purchased from AbD Serotech (Raleigh, NC). 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 TBS (-/-) for 5 minutes. Slides were blocked for 1 hour at RT in a blocking solution of 5% donkey serum. The buffer was then replaced with the primary antibody (diluted 1:100 in blocking solution) for 1 hour. Tissue samples were rinsed 3 times for 5 minutes with 1x PBS (-/-). Secondary antibody solution (diluted 1:100 in blocking solution) was then applied to the tissue samples and allowed to incubate for 1 hour in the dark. The slides were then rinsed with 1x TBS (-/-) in the dark for 5 minutes. Hoechst solution (1 mg Hoechst 33342 in 4 ml DI water, diluted 1:100 in 1x PBS (-/-)) was applied to the samples for 5 minutes. The slides were again washed for 5 minutes with 1x TBS (-/-) in the dark. Slides were mounted using FluorSave™ Reagent, and stored protected from light at 4 °C until imaging.
6.3.15. Histological Image Acquisition and Analysis

Images of H&E and Masson’s trichrome stained samples were collected using the 10x objective on a Nikon Eclipse TE2000-U with a Nikon Digital Sight DS-2Mv Digital Camera (Nikon Inc., Melville, New York). Images were taken by stitching individual 20x images to generate 6x6 large-image fields. Images of CD31 and CD68 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). Images were taken by stitching individual 20x images to generate 6x10 large-image fields. FITC filter was used to obtain the fluorescent images.

6.3.16. Collagen Capsule Density

As shown in Figure 16a, the density of the collagen in the capsule that formed around sensor implants was calculated by averaging four 50 x 50 μm² fields per tissue sample using a previously developed MATLAB (The MathWorks, Natick, MA) program. This program determines the percent collagen from images of Masson’s Trichrome stained tissue sections [35]. In short, 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. This ratio is then multiplied by 100 to obtain percent collagen for that field.

6.3.17. Microvessel and Macrophage Density

As shown in Figure 16b and 16c, the number of microvessels around implants was determined from CD31 stained sections, while the number of macrophages was determined from CD68 stained sections. For each image, a “ring-like” 100 μm wide zone beginning from the edge of the implant and moving outward was defined. Using ImageJ (National Institutes of Health, Bethesda, MD) this zone was selected and overlaid onto thresholded CD31 or CD68 images. Using the ‘analyze particles’ function the number of structures (count number) and the size of the selected zone in μm² were obtained. These measurements were then used to calculate the number of microvessels (CD31) or macrophages (CD68) per mm².

6.3.18. Sensitivity Measurements

Data from glucose sampling procedure were used to calculate sensor sensitivity over the duration of the study. Sensitivity for each sensor treatment was calculated based on the following formula:
\[ Sensitivity = \frac{I_i}{RGM_i} \]

Where RGM is the glucose reading from a conventional glucose meter (mg/dL) and I is the current measurement from the continuous glucose sensor (nA).

6.3.19. Mean Average Relative Difference (MARD)

Glucose Bolus data was be evaluated using the Mean Absolute Relative Difference (MARD) calculation. MARD is a mathematical calculation that measures the average disparity between the sensor and the reference measurement (blood glucose meters). The lower the MARD, the more accurate the device is considered. The MARD for a given interval was calculated using the following formula:

\[ MARD = \frac{1}{n} \left( \sum_{i=1}^{n} \left| \frac{CGM_i - RGM_i}{RGM_i} \right| \right) \]

Where n is the number of measurements, CGM is the blood glucose measurement from the implanted sensor and RGM is the reading from a conventional (reference) glucose meter.

6.3.20. Sensor Lag Time

Data from glucose bolus test were used to calculate changes in sensor signal lag. Sensor Lag time was calculated as the difference between the time at which glucose
concentration peaked in the blood and the time at which the current measurement peaked in the sensor. Sensor lag was calculated based on the following formula.

\[
Sensor \ Lag \ Time = CGM_{time} - RGM_{time}
\]

6.3.21. Statistical Analysis

All data is presented as means ± standard error of the mean. Data were tested for normality via Shapiro-Wilk tests, and quantile-quantile plots. Because the data were not normally-distributed, they were analyzed using the Wilcoxon Each Pair test. The Bonferroni procedure was applied to control the familywise error rate and to determine significance. Statistical significance was determined as \( p \leq 0.05 \).

6.4. Results:

6.4.1. H&E and Macrophage Analysis Healthy Rats

As shown in Figure 30, sensors deployed with Dex-loaded porous coatings showed decreased immune cell infiltration and migration to the implant site when compared to Dex-free porous coatings and bare controls at days 3 and 7 post-implantation. Inflammatory cell infiltrate was found surrounding the implants from the acute inflammatory phase at days 3 and 7. However, this effect decreased by day 21 when a thin-cellular layer similar to that of Dex-free implants formed around Dex-releasing
porous coatings. The heightened inflammatory state persisted through day 21 post-implantation in Dex-free implants. Furthermore, Figure 31 displays representative tissue sections of explanted sensor leads stained for macrophage specific marker CD68. By day 3 and 7 post implantation, a high density of CD68 positive cells was observed around Dex-free porous coatings and bare implants when compared the Dex-loaded porous implants. However, by day 21, a layer of CD68 positive cells had formed around Dex-loaded implants. Figure 32 displays the mean number of macrophages found within the 100 µm zone of the implant surface according to implant type and time of explantation. Dex-releasing porous coatings had significant lower macrophage densities at day 3 and 7 post-implantation, 33±6 and 40±7 cells/ µm² respectively, when compared to Dex-free porous and bare controls. However, by day 21 the number of macrophages around Dex-loaded implants increased resulting in no difference between the densities of macrophages around implants.
Figure 30. H&E stained images of Functional Sensor Implants and subcutaneous tissue after 3, 7 and 21 days in Rats. Bare and Dex-free implants show high levels of inflammation at days 3, 7 and 21. Sensors deployed with Dex-releasing porous coatings show decreased inflammation at days 3 and 7. By day 21, a thin ring of cells was visible on the surface of Dex-releasing porous coatings. Scale bars = 200 µm
Figure 31. CD68 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in Rats. High levels of CD68 positive cells were observed surrounding Dex-free percutaneous implants. Sensors deployed with Dex-releasing porous coatings had low levels of CD68 positive cells. Scale bars = 500 µm
Figure 32. Density of Macrophages within the 100 µm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in Rats. The tissues surrounding Dex-releasing porous coatings has significantly less macrophages than tissues surrounding Dex-free porous and Bare Sensor implants at days 3 and 7.
6.4.2. Collagen and Vessel Density Analysis Healthy Rats

Figure 33 shows the Masson’s trichrome stained images of bare, Dex-free porous and Dex-loaded porous coated functional sensors after 3, 7, and 21 days post-implantation. By day 21, bare implants show the formation of an organized collagen fibrils in direct apposition to the implanted sensor leads. Porous coatings show the formation of loose collagenous tissue that was well integrated into the surrounding subcutaneous tissue. Dex-releasing porous implants had similar response that of porous coatings showing disruption of collagen fibril formation.

Table 9. Collagen density in tissue adjacent to functional sensor implants in healthy rats (n=5 or greater).

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Percent Collagen (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
</tr>
<tr>
<td>Day 3</td>
<td>14.0 ± 9.5</td>
</tr>
<tr>
<td>Day 7</td>
<td>15.9 ± 10.2</td>
</tr>
<tr>
<td>Day 21</td>
<td>31.7 ± 8.1</td>
</tr>
</tbody>
</table>

As outlined in Table 9, there was no difference in collagen density between percutaneous implants at 3 and 7 days post-implantation. By day 21 the collagen density around porous
coated sensors regardless of Dex-loading was about 2-fold lower than that of bare sensors (about 16% collagen for porous coating and 30% for bare sensors).

Figure 34 displays representative CD31 receptor stained slides from explanted bare, Dex-releasing porous, and Dex-free porous coated glucose sensors at days 3, 7, and 21 post implantation. Little to no microvessels were observed near to the implants by day 3 post implantation. By day 7, tissue surrounding sensors coated with Dex-free porous coatings showed blood vessel formation. Moreover, enhanced blood vessels in the tissue near the implants was observed in sensors treated with porous coatings regardless of Dex-loading by day 21. Almost no vessels were observed in the tissue surrounding bare sensors by day 21. Also, it was observed that some vessels formed in the space between the sensor and the porous coatings however, this effect was random and found in a small subset of samples.

Figure 35 displays the mean number of microvessels found within the 100 µm zone away from the implant surface according to sensor treatment and time of experiment termination. Overtime, the number of vessels around porous coated implants increased regardless of Dex-loading. There was no differences in the vessel density between treatment types by day 3 post implantation with all implants having a mean vessel
densities between 20 and 30 vessels/mm². By day 7, however, Dex-free porous implants had a slightly higher vessel density (47±10 vessels/mm²) than bare and Dex-releasing porous coated sensors. By day 21, the tissue surrounding coated sensors regardless of Dex-loading showed a significantly higher vessel densities when compared to bare sensors.
Figure 33. Masson’s trichrome images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in healthy rats. By day 21, the formation of organized collagen capsule was observed on bare sensor implants. Porous implants regardless of Dex-loading were able to disrupt the formation of this capsule. Scale bars = 200.
Figure 34. CD31 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in healthy rats. No difference in vascularity between implants was observed for days 3 and 7. Dex-free Porous coated and Dex-Releasing porous coated sensors showed increased vascularity by Day 21. Scale bars = 500 µm
Figure 35. Density of blood vessels within the 100 μm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in healthy rats. The tissues surrounding implants showed no differences in vascularity at days 3 and 7. By day 21, vascularity for all porous implants regardless of Dex-loading had significantly increased when compared to controls.
6.4.3. Continuous Glucose Monitoring and Glucose Bolus Tests in Healthy Rats

Bare sensors and sensors coated with Dex-loaded porous coatings were implanted into the dorsal subcutis of rats for continuous, real-time glucose monitoring over 21 days. At least five out of seven sensors for each treatment group remained implanted until experiment termination. As shown in Figure 36, sensor sensitivities for all treatment groups were stable for the first ~ 10-15 days of the study. However, between days 10 and 15 the average sensor sensitivities from bare and Dex-free porous coated sensors decreased from ~ 0.2 nA/mg/dL of glucose to ~ 0.05 nA/mg/dL of glucose and stayed at those levels until experiment termination. Conversely, sensors treated with Dex-loaded porous coatings maintained high sensitivity levels (~0.2 nA/mg/dL) throughout the 21-day experiment. Moreover, sensors treated with Dex-loaded porous coatings had significantly higher sensitivity levels than Dex-free porous coatings and bare sensors from days 14 – 21 post-implantation.
Figure 36. Plot of sensor sensitivities of implanted Medtronic MiniMed SOF-Sensor glucose sensors. Dex-releasing porous coated sensors retained high sensitivity levels through the 21 day period when compared to Dex-free porous coated and bare controls. Sensitivity of Dex-free porous and bare controls decreased after day 12.
Representative bare, Dex-releasing porous and Dex-free porous coated sensor responses to intraperitoneal glucose bolus injections after 1, 3, 7, 14, and 21 post implantation are shown in Figure 37 (a-f). In general, it was observed that sensors were able to respond to glucose challenges at days 1, 3, and 7 regardless of treatment. A decline in signal in bare and Dex-free porous coated sensors was observed during glucose bolus test at day 14. By day 21, sensors bare and porous coated sensors did not respond to interstitial glucose challenges. Dex-releasing porous coated sensors remained functional through day 21 of testing.
Figure 37. Representative Glucose Bolus Tests of implanted Medtronic MiniMed SOF-Sensor glucose sensors at (a) 1 day, (b) 3 days, (c) 7 days, (d) 14 days, and (f) 21 days in healthy rats. Bare and Dex-free porous coated controls began to fail by day 14 post-implantation. Dex-Releasing porous coated sensors responded to glucose challenges through day 21 post-implantation. All coated sensors regardless of Dex-loading had increased sensor lag when compared to controls.
A lag between the time interstitial glucose levels peaked and the time the sensor current measurement peaked was detected in all coated sensors regardless of Dex-loading (Figure 37). Table 10 displays the calculated sensor lag times for glucose bolus tests. Sensor lag increased with time regardless of treatment. Typically, Dex-releasing and Dex-free porous coated sensors experienced ~ 12-20 min lag between peak tail vein blood glucose and sensor response, whereas a 3-12 min lag was observed for bare sensors.

Table 10. Sensor Lag during glucose bolus tests for healthy rats (n=5 or greater).

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sensor Treatment (Mean ± SEM)</th>
<th>Time Lag (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
<td>Porous Coating</td>
</tr>
<tr>
<td>Day 1</td>
<td>3 ± 3</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>Day 3</td>
<td>3 ± 3</td>
<td>12 ± 6*</td>
</tr>
<tr>
<td>Day 7</td>
<td>10 ± 6</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>Day 14</td>
<td>12 ± 8</td>
<td>22 ± 9*</td>
</tr>
<tr>
<td>Day 21</td>
<td>&gt; 60a</td>
<td>&gt; 60a</td>
</tr>
</tbody>
</table>

* Sensors failed before test
* denotes p<0.05 or less compared to Bare Sensor

Table 11 shows MARD values for all sensor treatments during glucose tests at days 1, 3, 7, 14 and 21. MARD values increased with time for all treatments. Sensors treated with porous coatings had higher MARDs than either bare or Dex-releasing porous
coatings for all time points. By day 21, Dex-free porous coated and bare sensors failed to respond glucose challenges, while Dex-releasing porous coated sensors responded to challenges but displayed high MARD values (~63%).

Table 11. Mean Average Relative Deviation during glucose bolus test for healthy rats (n=5 or greater).

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sensor Treatment (Mean ± SEM)</th>
<th>MARD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
<td>Porous Coating</td>
</tr>
<tr>
<td>Day 1</td>
<td>27.0 ± 7.6</td>
<td>32.4 ± 8.5</td>
</tr>
<tr>
<td>Day 3</td>
<td>18.9 ± 3.2</td>
<td>38.8 ± 11.1</td>
</tr>
<tr>
<td>Day 7</td>
<td>29.7 ± 4.1</td>
<td>34.4 ± 16.9</td>
</tr>
<tr>
<td>Day 14</td>
<td>52.5 ± 10.8</td>
<td>61.1 ± 14.4</td>
</tr>
<tr>
<td>Day 21</td>
<td>&gt; 100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Did not respond to glucose challenge

6.4.4. H&E and Inflammatory Cell Analysis Diabetic Rats

Figure 38 displays H&E results for functional glucose sensors deployed in diabetic rat tissues. Bare sensor implanted in rats showed a decreased immune cell infiltration and migration when compared to bare sensors implanted in healthy rats by day 3 (Figure 30). By day 21, a thin-cellular layer formed around all implants. Furthermore, Figure 39 displays representative diabetic tissue sections of explanted sensor leads stained for macrophage specific marker CD68. By days 3 and 7 post implantation, lower levels of
CD68 cells were observed around Dex-free porous coatings and bare implants when compared to the same implants and time points in healthy rats (Figure 31). However, by day 21, a layer of CD68 positive cells had formed around Dex-loaded implants. Figure 40 displays the mean number of macrophages found within the 100 µm zone of the implant surface according to implant type and time of explantation. We observed that the tissue surrounding bare and Dex-free coatings had lower macrophage densities at day 3 and 7 post-implantation, when compared to bare and Dex-free porous coated sensors in healthy rats (Figure 32). By day 21, however, the number of macrophages around all implants regardless of Dex-loading increased resulting in no difference.
Figure 38. H&E stained images of Functional Sensor Implants and subcutaneous tissue after 3, 7 and 21 days in diabetic rats. Scale bars = 200 µm
Figure 39. CD68 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in diabetic rats. Little to no macrophages were observed in the surface of percutaneous implants at days 3 and 7. However, by day 21 the number of Cd68 positive cells increased in all implants regardless of Dex-loading. Scale bars = 500 µm
Figure 40. Density of Macrophages within the 100 µm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in diabetic rats. Macrophage density increased with time for all implants regardless of treatment. Tissue surrounding Dex-releasing coatings had the lowest macrophage densities for all time points. Macrophage densities were on average lower in diabetic tissues when compared to healthy rats.
6.4.5. Collagen and Vessel Density Analysis Diabetic Rats

Figure 41 shows the Masson’s trichrome stained images of bare, Dex-free porous and Dex-loaded porous coated functional sensors after 3, 7, and 21 days post-implantation in diabetic rats. By day 21, there were no apparent differences in the response between bare implants, bare, Dex-free porous and Dex-loaded porous coated sensors. Unlike healthy rats, bare implants did not show the formation of an organized collagen fibrils in direct apposition to the implanted sensor leads, but rather experienced the formation of loose collagenous tissue. This same loose collagenous tissue was observed around Dex-free and Dex-loaded porous coatings.

Table 12. Collagen density in tissue adjacent to functional sensor implants in diabetic rats (n=3)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Percent Collagen (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
</tr>
<tr>
<td>Day 3</td>
<td>13.1 ± 12.5</td>
</tr>
<tr>
<td>Day 7</td>
<td>17.2 ± 13.6</td>
</tr>
<tr>
<td>Day 21</td>
<td>17.7 ± 14.1</td>
</tr>
</tbody>
</table>

As outlined in Table 12 there was no difference in collagen density between percutaneous implants at 3, 7 and 21 days post-implantation. In contrast with healthy
subjects the collagen density around bare functional sensor implants did not double from day 3 to day 21, but rather stayed ~13-18%. Moreover, lower collagen densities were not observed in the tissue surrounding Dex-free and Dex-loaded porous coatings when compared to bare implants at day 21.

Figure 42 displays representative CD31 receptor stained slides from explanted bare, Dex-releasing porous, and Dex-free porous coated glucose sensors at days 3, 7, and 21 post implantation in diabetic rats. Bare, Dex-free and Dex-loaded porous coatings did not experience an increase in blood vessel formation adjacent to the implant at any of the experimental time points. Furthermore, an approximately 100 µm thick vessel-free zone was observed around all implants regardless of treatment throughout the length of the study. When compared to normoglycemic tissues, diabetic tissues surrounding the Dex-free porous coated implants did not experience the same increase in CD31 expression at day 21 (Figure 43). Moreover, Dex-loaded porous coatings did not show the same levels of vascularization at day 21 post-implantation that were observed when the coatings were implanted in healthy subjects. Finally, no differences in the level of vascularization of the tissues surrounding implants was observed during any of the experimental time-point.
Figure 41. Masson’s trichrome images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in Diabetic Rats. By day 21, the formation of organized collagen capsule was not observed on bare sensor implants. Scale bars = 200 µm
Figure 42. CD31 images of Functional Sensor Implants and subcutaneous tissue after 3, 7, and 21 days in Diabetic Rats. No difference in vascularity between implants was observed for days 3, 7 and 21. Moreover, vascularity of diabetic tissue that surrounded implants was lower than the one presented in healthy rats. Scale bars = 500 µm
Figure 43. Density of blood vessels within the 100 µm zone surrounding implanted Medtronic MiniMed SOF-Sensor glucose sensors in healthy and diabetic rats at day 21 post-implantation. There was no differences between the vascularity around bare implants in healthy and diabetic rats. In diabetics, vascularity for all porous implants regardless of Dex-loading was lower when compared to healthy rat controls.
6.4.6. Continuous Glucose Monitoring and Glucose Bolus Tests in Diabetic Rats

Bare sensors and sensors coated with Dex-loaded porous coatings were implanted into the dorsal subcutis of diabetic rats for continuous, real-time glucose monitoring over 21 days. All sensors remained implanted until experiment termination. As shown in Figure 44, sensor sensitivities for all treatment groups were stable for the first 3-5 days of the study. However, between days 5 and 10 the average sensor sensitivities from bare and Dex-free porous coated sensors decreased from ~ 0.15 nA/mg/dL of glucose to ~ 0.05 nA/mg/dL of glucose and stayed at those levels until experiment termination. Conversely, sensors treated with Dex-loaded porous coatings maintained high sensitivity levels (~0.15 nA/mg/dL) until day 15. Though, Dex-loaded porous coated sensors maintained higher sensitivity levels throughout days these differences were not statistically significant.

Representative bare, Dex-releasing porous and Dex-free porous coated sensor responses to intraperitoneal glucose bolus injections after 1, 3, 7, 14, and 21 post implantation are shown in Figure 45 (a-f). In general, it was observed that sensors were able to respond to glucose challenges at days 1, 3, and 7 regardless of treatment. A decline in signal was observed in bare and Dex-free porous coated sensors during glucose bolus test at day 14. By day 21, sensors bare and porous coated sensors did not respond to
interstitial glucose challenges, while Dex-releasing porous coated appeared to remain functional. This results mirror those reported in healthy rats, where Dex-releasing porous coatings were able to respond to glucose challenges at later time points.

A lag between the time interstitial glucose levels peaked and the time the sensor current measurement peaked was detected in all coated sensors regardless of treatment. Table 13 displays the calculated sensor lag times for glucose bolus tests in diabetic rats. Sensor lag increased with time regardless of treatment. Typically, Dex-releasing and Dex-free porous coated sensors experienced ~17-23 min lag between peak tail vein blood glucose and sensor response, whereas a 5-12 min lag was observed for bare sensors. Similar trends were observed in normoglycemic subjects.

**Table 13. Sensor Lag during glucose bolus tests for diabetic rats (n=3).**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sensor Treatment (Mean ± SEM)</th>
<th>Time Lag (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
<td>Porous Coating</td>
</tr>
<tr>
<td>Day 1</td>
<td>5 ± 6</td>
<td>17 ± 10</td>
</tr>
<tr>
<td>Day 3</td>
<td>10 ± 5</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>Day 7</td>
<td>12 ± 4</td>
<td>16 ± 7</td>
</tr>
<tr>
<td>Day 14</td>
<td>15 ± 10</td>
<td>&gt;60 a</td>
</tr>
<tr>
<td>Day 21</td>
<td>&gt;60 a</td>
<td>&gt;60 a</td>
</tr>
</tbody>
</table>

* Sensors failed before test
Table 14 shows MARD values for all sensor treatments during glucose tests at days 1, 3, 7, 14 and 21 in diabetic subjects. MARD values increased with time for all treatments. Sensors treated with porous coatings had higher MARDs than either bare or Dex-releasing porous coatings for all time points. By day 21, Dex-free porous coated and bare sensors failed to respond glucose challenges, while Dex-releasing porous coated sensors responded to challenges but displayed high MARD values (~55%). Similar trends were observed in normoglycemic subjects.

Table 14. Mean Average Relative Deviation during glucose bolus test for diabetic rats (n=3).

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sensor Treatment (Mean ± SEM)</th>
<th>MARD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
<td>Porous Coating</td>
</tr>
<tr>
<td>Day 1</td>
<td>23.9 ± 13.2</td>
<td>39.3 ± 14.5</td>
</tr>
<tr>
<td>Day 3</td>
<td>22.5 ± 7.5</td>
<td>32.3 ± 16.5</td>
</tr>
<tr>
<td>Day 7</td>
<td>28.9 ± 6.8</td>
<td>33.3 ± 18.3</td>
</tr>
<tr>
<td>Day 14</td>
<td>60.5 ± 10.8</td>
<td>&lt; 100 a</td>
</tr>
<tr>
<td>Day 21</td>
<td>&lt; 100 a</td>
<td>&lt; 100 a</td>
</tr>
</tbody>
</table>

a Did not respond to glucose challenge
Figure 44. Plot of sensor sensitivities of Medtronic MiniMed SOF-Sensor glucose sensors implanted in diabetic rats. Sensitivity of the sensors decreases after 7 days of implantation regardless of treatment.
Figure 45. Representative Glucose Bolus Tests of Medtronic MiniMed SOF-Sensor glucose sensors implanted in diabetic rats at (a) 1 Day, (b) 3 Days, (c) 7 Days, (d) 14 Days, and (f) 21 Days. Porous coated sensors did not respond to glucose challenges starting on day 14. All coated sensors regardless of Dex-loading had increased sensor lag when compared to controls.
6.5 Discussion:

The adoption of continuous glucose monitoring platforms as the principal method for reporting of blood glucose measurements has been hindered due to the premature in vivo failure of such devices [7, 10, 24, 37]. Biocompatibility studies have shown that more often than not upon implantation a sensor is presented with a harsh ever-changing microenvironment that hinders its functionality. This reaction is known as the foreign body response and has been identified as the primary mechanism behind glucose sensor failure in vivo. Studies of this response have identified that the consumption of glucose by inflammatory cells and lack of vascularity around implanted sensors are key incidents during the FBR that cause premature sensor failure [25, 35, 114]. Therefore, a system that curbs inflammation while promoting the thorough vascularization of the tissue surrounding of implanted sensor leads would be desirable.

In Chapter 5, we reported on the in vivo testing characterization of Dex-releasing porous polyurethane coatings to improve the performance of glucose sensors [115]. This system aims at improving sensor function by combining angiogenic texturing with localized and timed release of a powerful anti-inflammatory agent (Dex). There, we demonstrated that Dex-releasing porous coatings were capable of curbing the initial inflammatory response, and promoting the formation of vascularized tissue around
Tygon® tubing implants. Employing a novel Type I diabetic model, we found relevant differences between the responses implantation of Dex-releasing porous coatings between healthy and diabetic rats. However, questions regarding the effects of coatings on sensor functionality still remained.

Gross histological observations showed that Dex-releasing coatings were capable of decreasing the inflammatory response to glucose sensor implantation at days 3 and 7 in healthy rats. However, by day 21 a ring of inflammatory cells had formed around glucose sensor implants (Figure 30). To further characterize this inflammatory state, immunohistochemistry was performed to identify and quantify the number of macrophages to the sensor surface (Figure 31 & Figure 32). As expected, Dex release from coatings significantly reduced the number of macrophages surrounding implanted sensors during early time points. However, when the Dex-payload had been fully deployed the number of macrophages increased and was no different than that of bare and Dex-free porous coated treated sensors. Interestingly, macrophage density remained elevated during the later time point for both bare sensors and sensors treated with Dex-free porous coatings. This elevated number of macrophages can attributed to the constant mechanic stimulation (i.e. micro motion) experienced by implanted sensor probes due to their percutaneous nature [34, 35]. Moreover, recent reports have highlighted how high
shear forces at the sensor-tissue interface can lead to a more aggressive FBR and lead to increased immune cell migration to the sensor surface [124, 125].

Collagen density and composition studies showed that the collagen density around bare sensor leads increased with time from 15% at day 3 to 31% by day 21 in healthy rats (Table 9). Though, Dex-releasing and Dex-free porous coated sensors had in average a lower collagen density than bare implants these differences were not statistically significant. Lower collagen density levels can be attributed to both Dex release and texturing effects. Glucocorticoids not only reduce immune cell recruitment but have been linked to the reduction of collagen production by fibroblasts. This Dex-induced inhibition of collagen production results from a receptor-mediated inhibition of gene expression of type 1 procollagen mRNAs [52, 60, 116]. Moreover, studies comparing collagen density around solid implants to porous implants of (PVA and ePTFE) noted that the density of the capsule that developed around the porous materials was much lower than that of solid materials [18]. Also, Braber et al., has shown that specific features in the size, shape of topographical cues alter the way fibroblasts orient and attach to substrates [117].

Significant differences in vascularity between coated and bare sensors were observed by day 21 post-implantation regardless of Dex loading in healthy rats (Figure
Increased vascularity can be attributed to the effects of controlled coating structure (porosity and pore size), and full deployment of Dex from coatings within (1-14 days post implantation). As previously reported, Dex-releasing and Dex-free porous coatings had a porosity of 90% and a pore size of 50-75 µm [115]. Several studies have reported on the angiogenic effects of texturing to increase vascularity around medical implants [18, 35, 63, 76, 115]. Though cellular pathways through which topographical cues promote angiogenesis and tissue integration, it is believed that pores within the 5-100 µm provide an optimal environment for transport of analytes and migration of endothelial cells [75].

Although both healthy and diabetic rats experienced an acute inflammatory response to functional sensor implants, the intensity of the response in hyperglycemic subjects is markedly decreased in comparison to healthy rats. The intensity of the inflammatory cell infiltration at the implant sites on days 3 and 7 in diabetic rats is much less than healthy rats. This decreased inflammatory response is caused by the altered immune cell function, cytokine production and cell metabolism characteristic of diabetic tissues. It has been reported that diabetic patients have a delayed immune response to tissue injury, pathogen or foreign body stimuli. This altered cytokine profile leads to deviations in the time and length of the inflammatory response and remodeling stages of the FBR.
When implanted in diabetic subjects, porous coatings regardless of Dex-loading were not able to promote the formation of a well vascularized tissue around functional sensor implants by day 21. These results were markedly different from those found in healthy rats, where porous coatings regardless of Dex-loading were capable of significantly increasing vessel density within the 100 µm zone when compared to controls. Differences in responses between diabetic and healthy subjects can be attributed to the reported lower levels or circulating EPCs in diabetic subjects, as well as the impaired homing of these cells to wound sites, when compared to normoglycemic tissues [95].

In contrast to functional sensor implants in healthy rats, implants in diabetic tissue did not show a pronounced increase in the collagen density over time. Collagen density of the diabetic tissue surrounding bare implants remained between 13 -18% while in healthy subjects it increased from 14%-32% from days 3 – 21 post-implantation. These noted differences in collagen deposition between diabetic and healthy tissues is due to the inhibited collagen synthesis by fibroblast found in hyperglycemic tissues [118, 119].

In both healthy and diabetic cases, increased sensor lag and MARDs scores for coated sensors regardless of Dex-loading when compared to non-porous controls were observed. This observation can be accredited to the augmented migration distance required for glucose to reach the sensor surface. Studies have shown that increasing the
distance that glucose has to migrate reach the sensor surface is the greatest determinant at increasing glucose sensor lag [81]. Moreover, *in vitro* functional sensor studies by Koschwanez et al. [35] similarly showed that sensors deployed with thick porous coatings (>200 µm) had elevated lag times when compared to thinner coatings (<100 µm).

Thought sensors treated with Dex-free porous coatings promoted the formation of a vascularized tissue network adjacent to the sensor by day 21 in healthy rats, they failed within 10 – 15 days of implantation. Similar findings were reported in studies where porous PLLA coated sensors resulted in a well vascularized microenvironment, yet this response did not translate into an extension of sensor functionality *in vivo* [34, 35]. Sensor failure was attributed to the effects of inflammatory cells on sensor function, therefore not only a vascularized tissue is necessary to guarantee adequate sensor function but also a mediation of the inflammatory stage.

Extended functionality of Dex-loaded porous coated sensors in healthy rats can be attributed to both the depletion of macrophages from the sensor surface as well as increased glucose transport via the enhanced vessel density of the tissue surrounding implanted sensor. Recent reports have suggested that macrophages not only induce premature sensor failure by enzymatic degradation and phagocytosis but rather impair sensor function via direct surface fouling, the secretion of cytokines that induce fouling
by neighboring cells, and by maintaining a metabolic state of high glucose consumption [30, 114]. After the full deployment of Dex from Dex-loaded coatings, topographical cues controlled the response and promoted the formation of a vascularized network by day 21. This vascular network allowed for the unimpeded transport of glucose to the sensor surface, which resulted in sensors remaining highly sensitive through the 21 day study. These results highlight the importance of mediating both the inflammatory and repair stages of FBR, to create a tissue microenvironment conducive to optimal sensor function.

Conversely, Dex-releasing porous coated sensors implanted in the dorsum of diabetic rats did not experienced the same extended functionality observed in sensors of similar treatment implanted in healthy rats. This difference in response can be attribute to the fact that porous coatings were not capable of significantly increasing vessel density within the 100 µm zone adjacent to implant when compared to controls.

6.6 Conclusions:

The development of coatings that can extend the functional life of implantable glucose sensors must address key aspects of the foreign body reaction such as inflammation and lack of capsule vascularity. This study evaluated the effectiveness of porous Dex-releasing coatings at modifying the tissue response to glucose sensor implantation and the subsequent effects on glucose sensor function using healthy and
diabetic rat animal models. Dex release from porous coatings attenuated inflammation induced by sensor implants, while topographical cues presented by coatings promoted the vascularization of the near-by tissue in healthy rats. The dual effect of diminished inflammation and increased vascularization of the tissue adjacent to sensors treated with Dex-releasing porous coatings allowed sensors to remain highly sensitive throughout the 21 day study in healthy rats. However, due to the increase in the glucose’s diffusion path, porous coated sensors regardless of Dex-loading experienced an increase in sensor lag and MARDs.

Differences between the wound healing of healthy and diabetic subjects was observed. Diabetic rats mounted a weak acute inflammatory response to sensor implantation, the number of inflammatory cells surrounding implants during days 3 and 7 post-implantation was reduced when compared to healthy controls. Evaluation of the angiogenic potential of porous coatings in diabetic animals showed that porous coatings regardless of Dex-loading were not able to promote the formation of a well vascularized tissue around implants. Due to the decreased vascularization of the diabetic tissue surrounding Dex-releasing porous coatings did not extend the functionality of implanted glucose sensors. Future work should focus on further studying sensor functionality in diabetic animal models, and developing approaches that not only hinder macrophage
migration and promote vascularity around sensor implants, but attempt to reduce glucose sensor lag.
7. Chapter 7: Overview, Conclusions and Future Work

7.1. Summary of Work

7.1.1. Porous, Dexamethasone-Releasing Coating Development and Characterization

Highly porous Dex-loaded coatings were fabricated with open architecture pores of cellular dimensions using the gas-foaming/salt-leaching technique. Dex release from coatings showed a typical initial fast release followed by a steady release with a high dependency in drug loading over a 15-day period. Porosity did not affect overall Dex release kinetics, however decreasing coating porosity increased sensor signal lag-time and attenuation. Therefore, 90% porous Dex-releasing coatings were determined to be best for further testing. Dex released from coatings was able to induced apoptosis of human derived peripheral blood monocytes in a time and dose dependent manner.

7.1.2. Tissue Response to Fully Subcutaneous Porous, Dexamethasone-Releasing Coatings in Healthy and Diabetic Tissues

Subcutaneously implanted Tygon® tubing implants that were either bare, coated in Dex-free porous coatings and Dex-releasing porous coatings. Dex-releasing porous coatings showed the anticipated effect attenuating initial inflammation via Dex release and impaired immune cell migration to implant site over a period of 14 days, while topographical cues were capable of enhancing vascularization around the implant by 21
days. Differences between healthy rats and diabetic subjects were observed. Though diabetic rats were able to mount an acute inflammatory response, the number of inflammatory cells surrounding implants was reduced when compared to healthy controls. Evaluation of the angiogenic potential of porous coatings in diabetic animal showed that porous coatings regardless of Dex-loading were not able to promote the formation of a well vascularized tissue around implants.

7.1.3. Tissue Response to Porous, Dexamethasone-Releasing Coatings and Subsequent Effects on Glucose Sensor Function in Healthy and Diabetic Rats

Medtronic MiniMed SOF-SENSOR™ glucose sensors were either bare, coated with Dex-free porous coatings or Dex-releasing porous coatings were implanted in the dorsum of healthy and diabetic rats. Dex release from porous coatings attenuated inflammation induced by sensor implants, while topographical cues presented by coatings promoted the vascularization of the near-by tissue in healthy rats. The dual effect of diminished inflammation and increased vascularization of the tissue adjacent to sensors treated with Dex-releasing porous coatings allowed sensors to remain highly sensitive throughout the 21-day study in healthy rats. However, due to the increase in the glucose’s diffusion path, porous coated sensors regardless of Dex-loading experienced an increase in sensor lag and MARDs. Differences between the wound healing of healthy and diabetic subjects
were observed. Diabetic rats were not able to mount an acute inflammatory response and the number of inflammatory cells surrounding implants was reduced when compared to healthy controls. Evaluation of the angiogenic potential of porous coatings in diabetic animal showed that porous coatings regardless of Dex-loading were not able to promote the formation of a well vascularized tissue around implants. We believe that due to the decreased vascularization of the diabetic tissue surrounding Dex-releasing porous coatings did not extend the functionality of implanted glucose sensors.

7.2. Conclusions:

The aims of this work have been to better understand how the foreign body response and wound healing contribute to glucose sensor failure, to design a system capable of modulating this response, and that such system would guide the creation microenvironment favorable for extended sensor function. To achieve these goals, a novel porous polyurethane coating that was capable of releasing Dex, to attenuate inflammation and present topographical cues in the cellular range to promote vascularization of the tissue surrounding implanted sensor leads was developed. The first study aimed at developing the coatings and characterizing their drug-releasing, and topographical properties. Most importantly, we showed that porous coatings did not interfere with sensor function in vitro and that Dex-release from the sensor surface was bioactive. We
further tested these coatings using healthy and diabetic rat animal models. We found that Dex-releasing porous coatings were capable of attenuating inflammation and promoting the formation of a vascularized tissue around subcutaneous implants. We also observed that there were differences between the healthy and diabetic wound healing response to coating implantation. Moreover, we found that coatings were capable of significantly improving the long-term sensitivity of coatings in a healthy rat. However, this improvement in glucose sensor sensitivity was accompanied with high MARD scores and a produced increase in signal lag.

7.3. Future Work:

7.3.1. Determine the Role of Macrophage Phenotype on Sensor Performance

A recent report by Novak et al. highlighted the deleterious effects that macrophages may have on sensor function by creating biomimetic gels that recreate the inflammatory state of the FBR [114]. However, the degree at which macrophages contribute to sensor failure may depend on their activation and subsequent macrophage phenotype. Classically activated macrophages (M1) secrete pro-inflammatory cytokines and play an important role in microbicidal activity, while alternative activated macrophages (M2) promote tissue repair and are considered immunomodulatory. Most importantly, M1 macrophages have a heightened metabolic state, which leads to a
condition of increased in glucose consumption when compared to a resting or M2 macrophage. Since an increase in glucose consumption at the sensor tissue interface may lead to an artificial decrease in the sensor signal, changes in metabolism between phenotypes have important ramifications for the functionality and accuracy of glucose sensors. Therefore, it will be important to characterize the phenotype of macrophages surrounding an implanted sensor lead.

One approach to characterize macrophage phenotype around implanted sensors would be to perform immunohistochemical assays on tissue sections of implanted sensors. First, a general macrophage specific marker (CD68) would be used to identify the target cell population. A secondary stain will then be used to identify M1 and M2 macrophages. It has been reported that M1 macrophages may express high levels of inducible nitric oxide synthase (iNOS), while the M2 phenotype is characterized by the expression of the mannose receptor C (CD206) [69].

Moreover, changes in macrophage phenotype could be further monitored using microdialysis. Macrophage phenotype can not only be determined by surface market staining but through the monitoring of cytokine production. M1 macrophages are characterized as producing high levels of pro-inflammatory cytokines, including interleukin IL-1β, IL-6, IL-12 and TNF-α [102, 126]. M2 macrophages are characterized by
producing high levels of the anti-inflammatory cytokine, IL-10, and low levels of inflammatory cytokines such as IL-1β, IL-6 and IL-12. By implanting microdialysis probes in the backs of rats one could monitor changes in the cytokine profile and relate such changes to the presence of a specific macrophage phenotype.

7.3.2. Role of Mechanical Irritation on Sensor Response

Though in this work we did not observe a striking differences between subcutaneous and percutaneous implants, recent reports have highlighted the deleterious effects of micro-motion may cause to sensor function [124, 125]. We hypothesize that we did not observe this micro-motion effects since the sensors in our studies were tightly secured to the backs of rats. In general, when fully implanted in the subcutis, subcutaneous implants form a dense, avascular capsule, in contrast similar implants may lead to a state of chronic inflammation in which the sensor in surrounded by granulation tissue.

Future studies could include investigating how mechanical irritation, micro motion and tissue damage created during sensor implantation influence the tissue response and affect sensor failure. One strategy will be to implant materials of varying stiffnesses either percutaneously and/or subcutaneously. Then the foreign body response
to these implants should be quantified by monitoring inflammation, macrophage density, vessel density, collagen capsule thickness, and collagen capsule density.

### 7.3.3. Novel Fully Implantable Sensor Technology

While percutaneous-amperometric glucose sensors have been the industry standard for the past 25 years. Novel sensing platforms have taken advantage of developments in biomaterials and biochemistry to give rise to new platforms and sensing modalities. Most importantly these novel sensors are characterized by having a fully implantable component, and an external detector that is not physically linked to the implanted monitor. For example, PROFUSA, Inc. is currently developing novel fully implantable glucose sensors [127]. Their system uses a porous pHEMA scaffold in which glucose oxidase (GOx) is immobilized. Unlike electrochemical sensors, this platform uses the optical signal from phosphorescence to detect changes in glucose concentration. As glucose is consumed by the immobilized GOx, local oxygen concentration is diminished, which results in a proportional increase in phosphorescence lifetime and intensity. Since these sensors use a light-based modality to detect changes in glucose, the implanted device can be monitored externally through the skin without the need of a physical transcutaneous connection to an instrument.
Moreover, the Maryland based company Senseonics recently reported on improved glycemic management by diabetics with the use of their fully implantable sensor system in patients with insulin-dependent diabetes[128]. Senseonics® Continuous Glucose Monitoring System uses a fluorescence-based fully implantable sensor that is 3 mm in diameter and 14 mm long. The implantable sensor has a polymer hydrogel film containing a proprietary indicator molecule that becomes fluorescent when it binds glucose. Detection of the fluorescent signal is done through an external miniature fluorometer that has a tiny LED for excitation. The implanted sensor is powered through an external body-worn reader that provides a wireless inductive RF signal. With all the advances in fully implantable glucose sensing technology, it will be insightful to apply our Dex-releasing coatings on these system to understand the benefits that localized Dex-release and enhanced tissue vascularization may carry.

7.3.4. Immunomodulatory Coatings

Recent advances in the fields of immunology and biomaterials have given rise to approaches that aim to control the FBR through the presentation of molecular mediators and surface cues that specifically regulate the immune system. In our study we observed that by completely attenuating the inflammatory response to sensor implantation, in combination of texturing cues that promote angiogenesis sensors remained highly
sensitive for the 21 day testing period. One of the limitations of this approach is that the systemic delivery glucocorticoids has been linked to an increase in blood glucose levels [60]. This would limit adoption of our Dex-releasing porous coatings on the diabetic population. Therefore, a more targeted approach that mediates the immune system to achieve the similar attenuation of inflammation in the short-term and promotion of angiogenesis in the long-term around implanted glucose sensors will be desirable.

Approaches that utilize specific molecular directors to target the inflammatory and regenerative phases of the foreign body response are currently being pursued. Successful decoupling has been achieved by Zachman et al. [50], where scaffolds capable of pro-angiogenic and anti-inflammatory actions were successfully developed. Porous scaffolds of tyrosine-derived polycarbonates were cross-linked with polyethylene glycol (PEG) dihydrazides, and fabricated into porous scaffolds by salt leaching. This scaffolds were then filled with a collagen gel. Pro-angiogenic and anti-inflammatory responses were activated by embedding functional peptides into a collagen gel. This collagen gel was then used as a vehicle for peptide delivery. They used Thymosin β-4-derived anti-inflammatory Ac-SDKP peptides that had been shown to decrease macrophage infiltration and TGF-β expression, in conjunction with Laminin-1-derived pro-angiogenic C16 peptides to promote endothelial cell (EC) adhesion, tube formation, and angiogenesis.
These two peptides were released in conjunction from the collagen gel embedded and were found to activate angiogenesis, but suppress the inflammatory response both \textit{in vitro} and \textit{in vivo}. However, when monitoring the secretion of cytokines from macrophages exposed to this two peptides, the treatment with the pro-angiogenic C16 peptide stimulated an increase in pro-inflammatory cytokine secretion (IL-1β, IL-6, IL-8, and TNF-α) relative to control, but anti-inflammatory Ac-SDKP treatment was able to diminish this response. Moreover, a simultaneous co-treatment with the two peptides did not change the cytokine levels as compared to Ac-SDKP treatment alone. Since \textit{in vitro} and \textit{in vivo} experiments show trends of a decreased anti-inflammatory state while still promoting angiogenesis when both peptides are released, the authors hypothesized that the co-treatment might direct a mechanism to reduce inflammatory responses, while maintaining pro-angiogenic capacity at the site.

Efforts to use topographical cues within the μm range as a mechanism to control macrophage phenotype without the delivery of bioactive agents have gained favor in the biomaterials community. These efforts have demonstrated to have large effects on guiding macrophage adhesion, fusion, and cytokine production. Buddy Ratner [75, 80, 102] and colleagues have developed a technique to create porous polymer constructs were every pore is the same size and porous interconnects are also uniform in size. These constructs
are referred to as porous template scaffolds (PTS) and are generated by fully packing poly(methyl methacrylate) (PMMA) of a desired diameter into a mold. Gently heating of the mold leads to the fusion of the spheres at their contact points. A monomer is then infused into the mold and later polymerized in-place into a solidified cross-linked network. Finally, the PMMA beads are solubilized from within the cross-linked network, leaving a porous, interconnected structure behind. In vivo studies using these scaffolds have demonstrated that rich macrophage infiltration in the 30-40 um pore PTSs, corresponded with increased vascular density of the implants. To determine the role that macrophages may have on this improved healing response, non-porous and 40 um porous PTSs were implanted in rat cardiac tissue for four weeks [69]. After this time, macrophage phenotype was determined by looking at nitric oxide synthase receptor and mannose receptor as pro-inflammatory (M1) and pro-healing (M2) markers respectively. Expression of M1 markers was about four fold higher than flat surfaces, while the number of cells expressing both M1 and M2 or only M2 markers was higher inside and around PTSs.
References


**Biography**

Suzana G. Vallejo-Heligon was born in Caracas, Venezuela in 1986. She attended elementary, middle and high school in El Colegio Claret before immigrating to the United States with her mother and sister in December of 2002. Once in the US, Suzana graduated from Gainesville High School in 2004. She attended college at the University of Florida where she received a Bachelor of Science in Materials Science and Engineering degree with a *magna cum laude* distinction in 2009. While at UF, Suzana developed her passion for biomedical engineering by engaging in numerous research projects across several laboratories. She was also a recognized leader of the Society for Hispanic Professional Engineers and a prominent member of the STEP-UP program at UF. Thanks to her stellar academic record, research contributions, and service to the college of engineering, Suzana was selected as the 2009 Female Speaker for the University of Florida Commencement Ceremony.

Suzana started the PhD Biomedical Engineering program at Duke University in 2009, under the mentorship and support of Dr. William “Monty” Reichert and received a M.S in Biomedical Engineering from Duke in the fall of 2012. Her research interest include biomaterials, biocompatibility, medical device design, and the commercialization of medical technologies. For her PhD research, she developed a novel coating to improve the
functional life of implantable glucose sensors for diabetes management. During her time at Duke, Suzana was a Lew fellow for the Center of Biomaterials and Tissue Engineering and the recipient of the Medtronic Fellowship. She was also an active member of the Engineering Graduate Student Council and a founding leader of the PhD Plus Certificate program. In her spare time, she enjoys hiking, camping, traveling, diving, and skiing.

Publications:


Oral Presentations:


Poster Presentations:


Awards:

Society for Biomaterials Student STAR Award – Society for Biomaterials 2015
Honorable Mention Society for Biomaterials Student STAR Award – Society for Biomaterials 2014
2014 Kewaunee Poster Session Senior Graduate Award – Center for Biomolecular and Tissue Engineering, Duke University
Lew Graduate Fellowship – Center for Biomolecular and Tissue Engineering, Duke University
Medtronic Graduate Fellowship – Biomedical Engineering Department, Duke University