A Dissertation Presented

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To my biggest cheerleaders: Mom, Dad, John, Pat and Liz
...here’s to leaning in
ABSTRACT

Diabetes is an international health epidemic characterized by hypo and hyperglycemic levels. To maintain glycemic control, diabetics test glucose levels several times a day. However, the current glucose monitoring approaches suffer from invasiveness, intermittent measurements, accuracy, and lifetime. This dissertation details the design of novel fluorescent glucose-sensitive sensors for monitoring glucose levels non-invasively and continuously after initial implantation. Sensing components were selected for appropriate response at physiological concentrations and were fully characterized for dynamic range, sensitivity, and lifetime in vitro. In preclinical testing, glucose-sensitive sensors tracked changes in glucose levels in mice, but sensor monitoring was limited to one hour. Sensor design was further improved after these studies to prolong in vivo lifetime, increase response at hypoglycemic levels, and enhance sensor biocompatibility. These efforts resulted in in vivo lifetimes greater than one hour, incorporation of more advance sensing moieties, and a biodegradable sensor platform. Future work with these sensors will involve Clark Error Grid Analysis and biodistribution studies to address clinical application requirements.
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LIST OF PUBLICATIONS AND PRESENTATIONS

Publications


Presentations


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Chapter 1: Dissertation Summary

1.1 Motivation: A Global Epidemic

Diabetes is a national and international epidemic. Currently, 26 million people in the United States, 1 92 million people in China, and 51 million people in India, are diabetic. An estimated 439 million people, 7.7% of the world population, will be diagnosed with diabetes by 2030. Diabetes is a chronic disease caused by elevated glucose levels and is linked to long-term effects such as heart disease, stroke, and blindness. Concurrently, diabetes’s prevalence, cost, and a battery of related health effects have spurred prevention programs, research into the disease, and monitoring devices. Monitoring glucose levels minimizes large and potentially deadly swings in glucose levels. The finger-prick method is the current gold standard for diabetics to check their glucose levels; however, this method results in patient non-compliance and intermittent measurements because of blood sampling requirements. Commercially available glucose monitors such as DexCom™ STS™ Continuous Glucose Monitoring System and Medtronic’s Guardian® REAL-Time Continuous Glucose Monitoring System were developed as minimally-invasive and continuous monitoring systems that are FDA approved to track trends in glucose levels, but still require blood measurements before therapeutic action is taken. Therefore, ongoing research into novel approaches for glucose monitoring is still of interest in order to prolong sensor lifetimes, improve accuracy, and minimize invasiveness of measurements.

1.2 Objective: Monitoring an Epidemic

The Clark group is developing a nanoclinical analyzer (NCA) that will monitor important physiological analytes such as glucose, sodium, and potassium. The NCA is a three-component system consisting of a minimally-invasive injection device, fluorescent nanosensors, and a
handheld optical reader (Figure 1.1). The fluorescent nanosensors will be placed into the upper layers of the skin with the injection device. As the concentration of the analyte of interest fluctuates, the nanosensors undergo a change in their fluorescence intensity. The handheld optical reader such as an iPhone interrogates these changes and provides the user with a measurement. With the NCA, users will be able to monitor analytes continuously and non-invasively after initial injection of nanosensors thus minimizing the need for painful blood draws. The focus of this dissertation is nanosensor development specifically for glucose. Fluorescent nanosensors are a novel approach for monitoring glucose levels in diabetes care. Their size imparts several advantages such as rapid response times, easy in vivo implantation, and sensing with nonbiological components making them ideal human use.

1.3 Dissertation Overview

This dissertation covers the main developments in glucose-sensitive nanosensor design. Several key questions guided the design of glucose-sensitive sensors and are the framework for this dissertation:

1) **Can glucose be extracted into a hydrophobic sensor using only non-biological components?** Traditionally, optode-based sensors have only detected and monitored ions because of difficulties extracting larger hydrophilic molecules into the lipophilic polymer matrix. Other glucose sensors utilize biological components such as enzymes, which while suitable for in vitro detection, pose problems for in vivo analysis. Chapter 2 covers the synthesis, characterization, and application of sensor components for reversible fluorescent responses to glucose extraction. Boronic acid compounds such as octylboronic acid were able to extract glucose from solution into the polymer core and reversibly bind to glucose.
Figure 1.1. Proposed *nanoclinical analyzer* (NCA). The NCA is a three component system containing (1) a minimally-invasive injection device, (2) fluorescent nanosensors, and (3) a handheld imaging device.
2) Can glucose nanosensors respond to physiological glucose levels? After selection of sensing components, glucose sensors were tailored to respond at physiological levels prior to in vivo experiments. Chapter 3 details full in vitro analytical sensor characterization. These nanosensors were the first demonstration of optode-based sensors for small molecules. With a K_d of 38 mM and response time of minutes, the formulation of sensors, while not optimal for physiological levels of glucose, are responsive enough in the physiological range for application.

3) Can glucose nanosensors be visualized through the skin and track changes in glucose levels in vivo? We performed initial animal testing in mice to determine if the sensors could be visualized through the skin before further optimization. We were concerned that excitation and emission wavelengths of our current fluorescent indicator (460nm and 570nm respectively) would not allow us to visualize the nanosensors over the autofluorescence of the skin. In addition to sensor characterization, Chapter 3 also demonstrates initial in vivo testing of glucose-sensitive nanosensors in mice. With the current sensor design, the nanosensors were visualized in the skin and additionally tracked changes in blood glucose levels when the mice were orally administered a glucose gavage.

4) How can nanosensor migration and cellular uptake be limited in vivo? Though initial in vivo testing of the glucose nanosensors was successful, our monitoring time was limited to an hour because of rapid migration and cellular uptake of the nanosensors from the site of injection. One straightforward approach that did not require dramatically changing the sensor itself was to encapsulate the sensors in injectable gels. Chapter 4 details encapsulation methods and gel retention of the nanosensors in vivo. In in vivo studies, the
gels significantly retained the nanosensors more than nanosensors without gels, but, there was still rapid loss of intensity over the course of hours.

5) **Will the sensors continue to function when fabricated with biocompatible materials?**

Nanosensor migration and cellular uptake not only hindered sensor monitoring time, but also raised toxicity concerns related to the ultimate fate of the sensors *in vivo*. Our current nanosensor was built on a poly(vinyl) chloride (PVC)/sebacate platform based on our history with ion-selective optodes,9,11 which have traditionally incorporated these materials. Though medical grade products contain both of these components, plasticizer leaching and known carcinogenic effects of vinyl chloride have caused rising safety concerns associated with plasticized PVC in medical devices.12,13 Although we have not seen any gross indications of biocompatibility issues, it was important to develop our nanosensors with known FDA approved materials before further animal testing and long-term biocompatibility studies.

Chapter 5 details our design of the first optode-based biodegradable nanosensors using polycaprolactone (PCL) and a citrate-based plasticizer. These studies were conducted with sodium nanosensors because their sensing mechanism is robust, mathematically described, and well characterized. The new PCL-based nanosensors yielded similar characteristics and analytical response to PVC-based nanosensors.

6) **Prior to further *in vivo* testing, can the dynamic range, sensitivity, and lifetime of the sensors be improved?**

Previously developed glucose-sensitive nanosensors (Chapter 3, question 2 above) have two key limitations independent of *in vivo* obstacles: leaching of sensing components and a dynamic range insensitive to hypoglycemic events. Chapter 6 describes the testing of alternative sensing moieties and fluorescent dyes for the glucose nanosensors. To prevent component leaching, alizarin derivatives were conjugated to the
polymer platform and tested for sensor response. Sensor response was also improved by incorporating boronic acid derivatives with functional groups that enhance glucose binding at physiological levels.

7) **Can changing sensor geometry prevent sensor migration and cellular uptake *in vivo***?

Though the gels (Chapter 4, question 4 above) decreased *in vivo* sensor migration, they were not a long-term solution to the problem of nanosensor migration because they only extended in vivo experiments to several hours. Electrospinning creates nanofibrous scaffolds that enlarge the overall sensing device to eliminate migration while retaining the high surface area to volume ratio characteristics of nanoparticles which is essential for the rapid response needed *in vivo*. As detailed in Chapter 7, electrospun nanofibers were fabricated using traditional glucose sensing components and yielded a response to glucose. Future work will involve testing this sensor platform *in vivo* to determine if it prolongs our *in vivo* monitoring time.

Figure 1.2 summarizes the progress and key milestones in glucose-sensitive sensor development. The dissertation concludes with future directions and recommendations for these sensors.
Figure 1.2. Schematic of dissertation overview detailing work completed, in progress, and future work. Major achievements and publications are also noted.
Chapter 2: Design and Development of Fluorescent Glucose-Sensitive Nanosensors

2.1 Chapter Overview

Background: The advent of fluorescent nanosensors has enabled intracellular monitoring of several physiological analytes which was previously not possible with molecular dyes or other invasive techniques. We have extended the capability of these sensors to include the detection of small molecules with the development of glucose-sensitive nano-optodes. Herein, we discuss the design and development of glucose-sensitive nano-optodes which have been proven functional both in vitro and in vivo.

Methods: Throughout the design process, each of the sensor formulations was evaluated based on their response to changes in glucose levels. The percent change in signal, sensor reversibility, and the overall fluorescence intensity were the specific parameters used to assess each formulation.

Results: A hydrophobic boronic acid was selected that yielded a fully reversible fluorescence response to glucose in accordance with the sensor mechanism. The change in fluorescence signal in response to glucose was approximately 11%. The use of different additives or chromophores did not improve the response; however, modifications to the plasticized polymeric membrane extended sensor lifetime.

Conclusions: Sensors were developed that yielded a dynamic response to glucose and through further modification of the components, sensor lifetime was improved. By following specific design criteria for the macrosensors, the sensors were miniaturized into nano-optodes that track changes in glucose levels in vivo.
2.2 Introduction

Current continuous glucose monitoring systems primarily rely on electrochemical biosensors using glucose oxidase and glucose dehydrogenase.\textsuperscript{14,15} These implantable sensors monitor glucose levels in the blood or interstitial fluid by measuring the oxidation of glucose in enzymatic reactions. Their long-term use, however, has been hampered by sensor degradation due to, in part, by foreign body responses at the sight of implantation.\textsuperscript{6,16,17} In an effort to improve sensor lifetime \textit{in vivo}, murine models for testing implantable glucose sensors have been developed to understand the mechanisms for sensor degradation.\textsuperscript{17,18} Though improvements have been made, the dependence of the sensor mechanism on enzymatic reactions and electrochemical readout still remains a limitation.

In response to the shortcomings of current techniques, researchers have developed alternative methods such as optical approaches for continuously monitoring glucose levels. For example, glucose sensing contact lenses that use photonic crystal sensors are being developed for non-invasive glucose monitoring.\textsuperscript{19,20} These sensors swell in the presence of glucose causing a shift in the diffraction wavelength of the sensor. Near-infrared spectroscopy can also be used to measure glucose non-invasively and has been successful in extracting glucose measurement information from transmission spectra across the human tongue.\textsuperscript{21} In other work, fluorescence resonance energy transfer (FRET) can report changes in glucose concentrations using a competitive binding assay encapsulated by a hydrogel particle.\textsuperscript{22,23} In our work, we present the use of glucose nano-optodes based ion-selective optode technology as a non-invasive glucose monitoring tool.

For the past four decades, ion-selective electrodes (ISEs) have been used to measure a variety of important physiological analytes.\textsuperscript{24} More recently, bulk optodes have been developed
as an optical counterpart to electronic ISEs. These optodes are composed of a plasticized polymeric membrane in which recognition elements, fluorescent indicators, and additives are encapsulated. Mechanistically, optodes function through the bulk extraction of an analyte into the membrane by the recognition element. This extraction causes a concentration change within the membrane and alters the optical signal of the optode. For example, in the case of ion-selective optodes, extraction of ions generates a shift in the pH within the membrane. In order to maintain the charge balance of the membrane, the protonation state of the chromophore changes resulting in a measurable change in the optical signal. Through the use of different recognition elements and fluorescent molecules, bulk optodes have been developed for measuring sodium, potassium, calcium, chloride, and a host of other analytes. Furthermore, this technology has been miniaturized and developed into fluorescent nanosensors which have enabled the intracellular measurements of several physiological analytes.

Previously, we have reported on the development of glucose-sensitive nano-optodes that extend ion-selective optode technology to the detection of small molecules. In our glucose nano-optodes, dynamic changes in glucose concentrations are monitored using a competitive binding scheme between a hydrophobic boronic acid recognition molecule, a chromophore, and glucose. In the absence of glucose, the boronic acid is bound to the diol-containing alizarin, generating a fluorescent complex (Figure 2.1). When glucose is introduced into the system, glucose binds with the boronic acid, displacing the alizarin which renders it non-fluorescent. Competitive binding of this sort has been used in the development of other saccharide sensors and exploits the well-established affinity of boronic acids for diol moieties. Unlike other assays using this principle, our sensor components are encapsulated in a hydrophobic polymeric membrane imparting such benefits as decreased interference from other
Figure 2.1. Representation of the sensor mechanism. At low glucose concentrations, the boronic acid is bound to alizarin generating a highly fluorescent species. As the concentration of glucose increases, glucose is extracted into the sensor displacing the alizarin resulting in a non-fluorescent species.
biomolecules, the use of non-biological components, and sensor reversibility. The sensors are miniaturized to the nanoscale where the response time is on the order of seconds to minutes and it is possible to implant the sensors into the skin much like a tattoo. Thus far, we have demonstrated the functionality of these sensors both in vitro and in vivo, and we discuss herein the detailed development of the sensor formulation and optimization.

2.3 Materials and Methods

**Materials.** Bis (2-ethylhexyl) sebacate (≥ 97.0%) (DOS), 2-nitrophenyl octyl ether (≥ 99.0%) (NPOE), dipentyl phthalate (≥ 99.0%) (DPP), tris (2-ethylhexyl) phosphate (≥ 99.0%) (TBAI), tetrabutylammonium bromide (≥ 99.0%) (TBAB), tetrabutylammonium chloride (≥ 99.0%) (TBAC), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (≥ 98.0%) (EDC·HCl), poly (vinyl chloride) (high molecular weight) (PVC), and polyurethane (PUR) were all obtained from Fluka (St. Louis, MO). Alizarin, tetrahydrofuran anhydrous (≥ 99.9%) (THF), D-(+)-glucose (ACS Reagent Grade), dichloromethane (DCM), thionyl chloride (SOCl₂), pyridine (PY), and 2-propanol (IPA) (≥ 99.5%) were purchased from Sigma-Aldrich (St. Louis, MO). 4-mercaptophenylboronic acid (90%), 3-octanone, polycaprolactone (PCL), octylamine (99%) and 7, 8-dihydroxy-4-methylcoumarin (97%) were purchased from Aldrich (St. Louis, MO). Octylboronic acid (> 97%) was obtained from Synthonix, Inc. (Wake Forest, NC). Phosphate buffered saline (PBS) (1×, pH = 7.4) was purchased as a solution from Invitrogen (Carlsbad, CA). N,N-dimethylformamide (≥ 99%) (DMF) and alizarin-3-methyliminodiacetic acid were acquired from Sigma (St. Louis, MO). Poly(vinylidene chloride/acrylonitrile) (80:20) (P(VDC/AN) was purchased from Polysciences, Inc. (Warrington, PA) and α,ω-dicarboxy
terminated poly(methyl methacrylate) (PMMA(COOH)$_2$) was obtained from Polymer Source Inc. (Montreal, Canada). Commercially available materials were used without further purification.

**Polymer Composition of the Optode.** The optode from which the glucose sensors are made contains five main components: a polymer, plasticizer, boronic acid derivative, a chromophore, and an additive. The design of the sensor was obtained by optimizing each component. In all cases, the basic polymer optode was made from the following components: 30 mg of polymer, 60 µL of plasticizer, a boronic acid derivative, an additive, and a chromophore (see Tables 2.1-2.5). These materials were charged into a glass vial and then dissolved in 500 µL of THF. All formulations listed in Tables 2.1 through 2.5 formed optodes. Formulations that did not form optodes such as those containing poly(methyl methacrylate) have been excluded from the tables. Of note, composition is reported in mass as is standard in optode formulations.

**Chromophore Synthesis.** Compound A was prepared by initially treating ARS with SOCl$_2$ at 40 °C (Figure 2.2). After 65 hours, the reaction was cooled and PY and IPA were added directly to the mixture. The flask was then heated at 65 °C for an additional 18 hours. Upon cooling and removal of all volatiles, a brown powder was yielded. The crude product was used directly without further purification.

Compound B was prepared via reacting EDC·HCl with alizarin-3-methyliminodiacetic acid followed by the addition of octylamine at room temperature (Figure 2.3). After 12 hours, solvents were removed and the crude product was used without further purification.

**Macrosensor Response to Glucose.** The fluorescence data was acquired on a Spectramax Gemini EM microplate fluorometer (Molecular Device, Sunnyvale, CA). The excitation and emission wavelengths for the chromophores are listed in Table 2.5. Optode (2 µL) from each
Table 2.1. Composition of optodes with different boronic acids.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Plasticizer</th>
<th>Boronic Acid</th>
<th>Additive</th>
<th>Chromophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC</td>
<td>NPOE</td>
<td>1 mg 4-mercaptophenylboronic acid</td>
<td>0.5 mg TDMAC</td>
<td>1 mg alizarin</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>5 mg 2-ethoxypyridine-3-boronic acid</td>
<td>1 mg TDMAC</td>
<td>0.5 mg alizarin</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1 mg octylboronic acid</td>
<td>1 mg TDMAC</td>
<td>1 mg alizarin</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>1 mg 3-aminophenylboronic acid</td>
<td>0.5 mg TDMAC</td>
<td>1 mg alizarin</td>
</tr>
</tbody>
</table>
Table 2.2. Composition of optodes with different additives.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Plasticizer</th>
<th>Boronic Acid</th>
<th>Additive</th>
<th>Chromophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC</td>
<td>NPOE</td>
<td>1 mg octylboronic acid</td>
<td>0.5 mg TBAC</td>
<td>1 mg alizarin</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.5 mg TBAB</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.75 TBAI</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1 mg TDMAC</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Table 2.3. Composition of optodes with different polymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Plasticizer</th>
<th>Boronic Acid</th>
<th>Additive</th>
<th>Chromophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC-COOH</td>
<td>DOS</td>
<td>3 mg octylboronic acid</td>
<td>4 mg TDMAC</td>
<td>1 mg alizarin</td>
</tr>
<tr>
<td>PVC</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>P(VDC/AN)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>PMMA(COOH)$_2$</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>PCL</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>PUR</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Table 2.4. Composition of optodes with different plasticizers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Plasticizer</th>
<th>Boronic Acid</th>
<th>Additive</th>
<th>Chromophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC-COOH</td>
<td>DOS</td>
<td>3 mg octylboronic acid</td>
<td>4 mg TDMAC</td>
<td>1 mg alizarin</td>
</tr>
<tr>
<td>&quot;</td>
<td>NPOE</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>DPP</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3-octanone</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>TEP</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Table 2.5. Composition optodes with different chromophores.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Plasticizer</th>
<th>Boronic Acid</th>
<th>Additive</th>
<th>Chromophore</th>
<th>Excitation / Emission Wavelengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC-COOH</td>
<td>DOS</td>
<td>1 mg octylboronic acid</td>
<td>1 mg TDMAC</td>
<td>1 mg alizarin</td>
<td>460 / 570</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1 mg 7,8-DHMC</td>
<td>360 / 470</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1 mg ARS</td>
<td>420 / 590</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1 mg Compound A</td>
<td>420 / 590</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1 mg Compound B</td>
<td>460 / 570</td>
</tr>
</tbody>
</table>
Figure 2.2. Synthesis of Compound A.
Figure 2.3. Synthesis of Compound B.
formulation listed above was pipetted onto a glass cover slip on the bottom of a 96-well optical bottom well-plate. The optodes were allowed to dry for at least 15 minutes forming macrosensors. After drying, each optode was hydrated in 200 µL of PBS (pH = 7.4) for at least 1 hour in order to equilibrate the sensors with the surrounding aqueous solution. After the optodes were hydrated, the PBS solution was removed from all wells and 200 µL of 1 molar (M) (18,000 mg/dl) glucose in PBS (pH=7.4) was added to the experimental wells and 200 µL of fresh PBS was added to the control wells. High glucose concentrations were used to obtain the maximum fluorescence response from the sensors. Fluorescence measurements were taken with a 360 µs acquisition time at a sampling rate of 13 acquisitions per hour. The fluorescence response of the sensors was tracked for at least 60 minutes, at which time the fluorescence signal leveled off.10 At the end of this period, both the PBS and 1M glucose solution were removed and 200 µL of fresh PBS was added to all wells. Fluorescence measurements were acquired again in 360 µs acquisition times at a sampling rate of 13 acquisitions per hour. For each macrosensor, an average intensity was calculated from the final 2-3 intensity readings after the signal leveled off. This average was normalized to the average intensity reading from the initial solution change. By normalizing the intensities of each sensor, the responses between the control and experimental wells and also between different sensor formulations can be compared. The normalized values for each sensor were then averaged for both the control and glucose wells, respectively. The percent change in fluorescence response was then determined as the difference between the average normalized values. This value, which is typically negative, was inverted for purposes of presenting a clear graphical representation of the data.

2.4 Results
Sensor Response and Reversibility. In our sensors, boronic acid derivatives are the main recognition elements and are responsible for the dynamic fluorescence response to glucose. Due to its importance, boronic acid selection was the starting point for the glucose sensor design. Figure 2.4 shows the percent change in fluorescence response and reversibility for macrosensors containing different boronic acids. Each sensor formulation showed a response to glucose. 2-ethoxypyridine-3-boronic acid had the greatest percent change of 30.7 ± 0.6 %, but only sensors with octylboronic acid demonstrated almost full fluorescence reversibility as their fluorescence intensities recovered back to baseline after glucose was removed. Since octylboronic acid yielded a response complying with the sensor mechanism, all further sensor formulations used this boronic acid as the main sensing component.

Though octylboronic acid yielded a reversible fluorescence response to glucose, further modifications to the design were necessary to increase the percent change in fluorescence upon addition of glucose. We explored the use of several additives which could aid in the extraction of glucose into the sensor. TBAC and TDMAC showed the two greatest responses to changes in glucose concentrations with 10.8 ± 1.0 % and 11.3 ± 0.2 % decrease, respectively (Figure 2.5). TDMAC with its extended carbon chains was selected as the additive because we speculated that it would be less predisposed than TBAC to leach out of the sensor.

Fluorescence Intensity Optimization. Sensors composed of the traditional plasticized-PVC based polymeric membrane yielded the desired response to glucose; however, these sensors began to lose their fluorescence intensity immediately upon hydration (data not shown). Since the octylboronic acid and alizarin combination followed the sensor mechanism, changing the plasticized polymeric membrane was the initial method used to stabilize the fluorescence intensity and improve sensor lifetime. Substituting PVC-COOH for PVC drastically improved
Figure 2.4. Reversibility of the glucose macrosensors composed of different boronic acids. Each cycle represents a time frame of at least 1 hour. The macrosensors contained either 4-mercaptophenylboronic acid (■, $n_{control}=7$ and $n_{glucose}=7$), 2-ethoxypyridine-3-boronic acid (●, $n_{control}=6$ and $n_{glucose}=7$), octylboronic acid (▲, $n_{control}=8$ and $n_{glucose}=8$), or 3-aminophenylboronic acid (谤, $n_{control}=8$ and $n_{glucose}=8$).
Figure 2.5. Percent change in fluorescence of the macrosensors in response to glucose after at least 1 hour. The macrosensors contained either the additive TBAC ($n_{\text{control}}=7$ and $n_{\text{glucose}}=8$), TBAB ($n_{\text{control}}=8$ and $n_{\text{glucose}}=8$), TBAI ($n_{\text{control}}=8$ and $n_{\text{glucose}}=8$), or TDMAC ($n_{\text{control}}=8$ and $n_{\text{glucose}}=8$).
sensor lifetime. These sensors not only maintained their fluorescence intensities for up to 18 hours (data not shown), but they also yielded the greatest response (10.8 ± 0.4 %) to glucose after this time period (Figure 2.6). In contrast to changing the polymer, dramatic differences in response were not seen with a variety of plasticizers (Figure 2.7); however, DOS was chosen as the plasticizer because it yielded the greatest relative change.

Similar to the selection of the boronic acid, proper selection of the fluorescent indicator or chromophore is important because of the role it plays in the sensing mechanism. Alizarin’s fluorescence response was sufficient for the purpose of optimizing the other sensor components on the macroscale, but significant loss of fluorescence intensity occurs during sensor miniaturization. Therefore, hydrophobic high quantum yield fluorescent indicators are ultimately desired for sensor miniaturization. Alizarin derivatives and other diol chromophores were substituted for alizarin in the sensor formulation and were evaluated for maximum glucose response and maximum fluorescence intensity (Figure 2.8). Macrosensors with alizarin still had the greatest response to glucose with a percent change of 21.1 ± 0.6 %.

2.5  Discussion

For an effective in vivo glucose monitoring tool, the sensors must (1) be reversible in order to measure dynamic changes in glucose concentration, (2) be sensitive to small changes in glucose concentrations, (3) have a red shifted, high fluorescence intensity that can be measured through the skin, and (4) have hydrophobic components that will not leach out of the sensor. These four parameters were considered for each screened sensor formulation; however, particular parameters were emphasized depending upon the sensor component being tested.
Figure 2.6. Percent change in fluorescence of the macrosensors in response to glucose after at least 1 hour. The macrosensors contained either the polymer PVC-COOH (n_{control}=7 and n_{glucose}=6), PVC (n_{control}=7 and n_{glucose}=8), P(VDC/AN) (n_{control}=9 and n_{glucose}=9), PCL (n_{control}=6 and n_{glucose}=6), PUR (n_{control}=7 and n_{glucose}=8), or PMMA(COOH)$_2$ (n_{control}=6 and n_{glucose}=6).
Figure 2.7. Percent change in fluorescence of the macrosensors in response to 1 M Glucose in PBS after at least 1 hour. The macrosensors contained either the plasticizer DOS (n_control=7 and n_glucose=6), NPOE (n_control=8 and n_glucose=8), DPP (n_control=8 and n_glucose=8), 3-octanone (n_control=8 and n_glucose=8), or TEP (n_control=8 and n_glucose=8).
Figure 2.8. Percent change in fluorescence of the macrosensors in response to glucose after at least 1 hour. The macrosensors contained either the chromophore alizarin ($n_{\text{control}}=4$ and $n_{\text{glucose}}=3$), ARS ($n_{\text{control}}=8$ and $n_{\text{glucose}}=8$), 7, 8-DHMC ($n_{\text{control}}=3$ and $n_{\text{glucose}}=3$), Compound A ($n_{\text{control}}=8$ and $n_{\text{glucose}}=8$), or Compound B ($n_{\text{control}}=8$ and $n_{\text{glucose}}=8$).
As is shown in Figure 2.4, all the boronic acids bound glucose causing a decrease in fluorescence intensity but only sensors with octylboronic acid recovered their fluorescence intensity fully. The inability of these sensors to yield a reversible response may have been a result of leaching of the sensor components, as was the case for the hydrophilic molecule, 2-ethoxypyridine-3-boronic acid. This point emphasizes that hydrophobic components are essential for proper sensor function.

The sensitivity of the sensors can be improved through effective extraction of glucose into the sensor. In previous work on ion-selective optodes, the selectivity for specific analytes relied on the proper choice of the sensing molecule and the additive was included only to maintain electroneutrality.\textsuperscript{25} In the case of the glucose sensors, the additive was used to aid in the phase transfer of glucose into the sensor. Additives based on tetraalkylammonium salts, especially quaternary ammonium chloride salts, have been shown to be effective in extracting saccharides from the aqueous phase into plasticized polymeric membranes.\textsuperscript{38,39} Our results support these previous findings with sensors containing additives, TBAC or TDMAC, yielding the greatest percentage change in fluorescence intensity.

The response and lifetime of the sensors was improved by exploring different polymers. Substituting PVC-COOH for PVC stabilized the fluorescence signal while still generating a response to glucose. We theorize that the carboxyl groups help to maintain alizarin within the sensor and aid in the extraction of glucose. The mechanisms governing this and how the plasticizer, DOS, caused a greater sensor response is currently under investigation.

The chromophore also plays a critical role in the sensor mechanism and response. Previous research discussed above has shown that ARS can competitively bind with boronic acids in the presence of sugars.\textsuperscript{33-35} We explored using ARS as well as hydrophobic analogs of
this chromophore that could be maintained within the sensor. Sensors containing alizarin demonstrated the greatest percent change in fluorescence when in the presence of glucose and their fluorescence intensity remained stable over time. Other important selection criteria for the chromophore were that sensors had to have high fluorescence intensity and an emission spectrum in the near-infrared for optimal imaging of the sensors efficiently above the auto-fluorescence of the skin. For example, 7, 8-DHMC yielded significantly higher fluorescence intensities than alizarin, but its spectrum is blue-shifted, making it difficult for in vivo imaging. Alizarin’s emission spectrum is centered around 570 nm, and we have demonstrated previously that these sensors can be imaged above the auto-fluorescence of mice skin.

Proper selection of the sensor components not only determines the sensor functionality, but also extends sensor lifetime and stability. In order to achieve these, a major theme throughout the design process was the selection of hydrophobic sensing components which can be maintained within the sensors. After PVC-COOH was incorporated into the sensor, leaching was reduced in the macrosensors. However, miniaturization of the sensors into nano-optodes accelerates component leaching because of the increase in the surface area to volume ratio of the nano-optodes. This leaching results in decreased sensitivity and eventual loss of sensor functionality. As can be seen from the selection of the sensor components, they all exhibit some degree of hydrophobicity, but exploring more hydrophobic components would likely improve sensor lifetime in vivo.

2.6 Conclusions

Glucose macrosensors based on ion-selective optode technology were designed that are capable of monitoring dynamic changes in glucose levels. We have chosen the sensor
components primarily based on their ability to improve sensor response and fluorescence reversibility. The sensor formulation containing octylboronic acid, alizarin, TDMAC, DOS, and PVC-COOH fulfilled these design criteria and from this optode formulation, glucose nano-optodes were developed and tailored to respond within the physiological range. Furthermore, the nano-optodes from this formulation were able to track changes in glucose levels in vivo. These results are promising but further optimization of the sensors such as incorporating an internal reference dye, improving sensor sensitivity, and enhancing sensor biocompatibility are crucial developmental steps toward ultimate use in a clinical setting.


2.7 Acknowledgements

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Chapter 3: *In Vitro* Characterization and *In Vivo* Demonstration of Glucose-Sensitive Nanosensors

3.1 Chapter Overview

We have designed fluorescent nanosensors based on ion-selective optodes capable of detecting small molecules. By localizing the sensor components in a hydrophobic core, these nanosensors are able to monitor dynamic changes in concentration of the model analyte, glucose. The nanosensors demonstrated this response *in vitro* and also when injected subcutaneously into mice. The response of the nanosensors tracked changes in blood glucose levels *in vivo* that were comparable to measurements taken using a glucometer. The development of these nanosensors offers an alternative, minimally-invasive tool for monitoring glucose levels in such fields as diabetes research. Furthermore, the extension of the ion-selective optode sensor platform to small molecule detection will allow for enhanced monitoring of physiological processes.

3.2 Introduction

The introduction of fluorescent nanosensors, or PEBBLEs, made possible the intracellular analysis of a range of analytes which was previously impractical with molecular indicators. Based on ion-selective optode technology, nanoscale PEBBLEs were produced that could measure a large host of physiological parameters, including sodium, potassium and chloride. Further refinements of the sensors led to better stability and biocompatibility necessary for intracellular measurements.
Extending this nanosensor approach beyond detection of ions to small molecules has several limitations. Recognition elements for small molecules are traditionally based on biological reagents, such as enzymes (e.g. glucose oxidase\textsuperscript{43,44} or urease\textsuperscript{45}) or non-enzymatic proteins (e.g. antibodies or Concanavalin A\textsuperscript{22}). Although these sensors function adequately when applied on large length scales, when the size of theses sensors is radically scaled down, shortened lifetimes can occur due to regional depletion of resources, such as oxygen in the case of glucose oxidase function.\textsuperscript{43,44} In addition, since a limited number of biological elements are contained in the small sensor volume employed, any degradation greatly diminishes the function of the sensor.

When encapsulating an enzyme, the choices of polymeric shells are limited to hydrophilic components, such as hydrogels, in order to maintain function. As shown with ion-selective optodes, though, there are several advantages to using a lipophilic system for nanosensor development. First, it greatly expands the range of measurable analytes by enabling the use of a wider variety of chemistries. Second, by separating all of the lipophilic molecular components in the nanosensors from the bulk, aqueous solution, non-specific binding to proteins can be reduced.\textsuperscript{29} Third, the lipophilic core maintains these components at the proper concentrations leading to improved lifetime and stability of the nanosensors.\textsuperscript{9} Fourth, keeping the component in close proximity allows a completely reversible response of the nanosensors to dynamic changes in ion or small molecule concentration. Finally, the lipophilic environment makes possible an easily functionalized and tunable system. By adjusting the concentration of molecular components, the nanosensors can be tuned to respond within the ideal dynamic range and due to the ease of adding biocompatible surface coatings, these nanosensors can be made compatible with an intracellular environment and experience minimal aggregation.\textsuperscript{9,32}
Building on this lipophilic nanoscale platform, we introduce the first example of this class of optode-based nanosensor for small molecule recognition utilizing non-biological components. As a model analyte we demonstrate the detection of glucose. The nanosensor responds to glucose through the use of lipophilic boronic acid (BA) derivatives which have been extensively characterized as glucose recognition molecules. Other researchers have exploited the affinity of BA for glucose to design a variety of glucose sensors. Asher et al., for example, have developed photonic crystals that swell in the presence of glucose. Through measuring the light diffraction properties of the crystal, the glucose concentration can be inferred. Our chemistry, however, relies on a competitive binding mechanism between a lipophilic BA and a chromophore, alizarin. Glucose is reversibly extracted into the sensor by a BA in a similar fashion as previously reported for ion-based sensors. As depicted in Figure 3.1, alizarin, a non-fluorescent compound, reacts with a BA to produce a boronate ester, 1. Importantly, 1 is highly fluorescent and favored in the chemical equilibrium; therefore, the dissociation of 1 can be monitored by the loss of fluorescence intensity. When glucose is introduced into the equilibrium, a shift from the fluorescent species, 1, to glucose-derived boronate ester, 2, and non-fluorescent alizarin is observed. Therefore, the change in fluorescence intensity corresponds to the glucose concentration. A similar scheme has been used by James and Fossey to detect glucose using a solution phase assay and hydrogels, respectively. However, as previously mentioned, our system entraps the sensor components within the lipophilic core maintaining these components at the ideal sensing ratio.

Glucose plays a critical role in the body’s metabolism and dysfunction of glucose handling from insulin deficiency or resistance can lead to diabetes. Thus, the detection and monitoring of glucose has been the focus of diabetes-related research and technology. For this...
Figure 3.1. Chemical equilibrium of boronate formation between a boronate ester, 1, and glucose to form a glucose-derived boronate ester, 2.
reason, glucose was chosen as the model analyte for our nanosensor because of the applicability of the nanosensors as both a research and diagnostic tool.

### 3.3 Materials and Methods

**Materials.** Poly(vinylchloride) carboxylated (>97% GC), bis-(2-ethylhexyl)sebacate, tridodecylmethylammonium chloride and alizarin were purchased from Fluka (St Louis, MO, USA). D-(+)-glucose (ACS reagent grade), uric acid (≥ 99%, crystalline), acetaminophen (≥ 99%) and L-ascorbic acid (≥ 99%, crystalline) were obtained from Aldrich Chemical Co (St Louis, MO, USA). Octylboronic acid (>97%) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-550] (PEG lipid) in chloroform (10 mg/ml) were acquired from Synthonix (Wake Forest, NC, USA) and Avanti Polar Lipids, Inc. (Alabaster, AL, USA), respectively. Phosphate Buffered Saline (PBS) (1x, pH = 7.4) was purchased as a solution from Invitrogen (Carlsbad, CA, USA). Tetrahydrofuran (≥ 99%) (THF) was acquired from Sigma (St Louis, MO, USA). Chloroform (HPLC grade) was purchased from JT Baker (Phillipsburg, NJ, USA). Commercially available materials were used without further purification. CD1 mice were obtained from Charles River Laboratories International, Inc (Wilmington, MA, USA) and weighed 20-25 g.

**Polymer Composition of the Optode.** The polymer optode was made from the following components: 30 mg high molecular weight carboxylated poly(vinylchloride), 60 μl bis-(2-ethylhexyl)sebacate, 3.0 mg octylboronic acid, 4.0 mg tridodecylmethylammonium chloride, and 1.0 mg alizarin. These materials were charged into a glass vial and then dissolved in 500 μl THF. The vial was vortexed and the resulting optode was an orange solution.
Calibration of the Macrosensor. Data was acquired in a Spectramax Gemini EM microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). Optode (2 μl) from a bulk batch containing four times each of the components listed above was pipetted into 33 wells of a 96-well optical bottom plate each containing a glass cover slip. The optodes were then allowed to dry at least 15 minutes forming macrosensors. Each optode was hydrated in 200 μL PBS (pH=7.4) for at least 4 hours until the fluorescence signal stabilized. At the end of this period, the PBS solution was removed and another 200 μL of fresh PBS was added to the wells. After 40 minutes, an endpoint measurement was collected via exciting at 460 nm and emitting at 570 nm with a cutoff of 515 nm. The PBS solution was then replaced with 200 μl of 1 mM glucose in PBS (pH = 7.4). The optodes were allowed to equilibrate for 40 minutes, followed by an endpoint measurement. The process was repeated for 2 mM, 3 mM, 5 mM, 8 mM, 10 mM, 25 mM, 50 mM, 100 mM, 500 mM, and 1 M glucose in PBS. Response was determined by expressing the data as \( \alpha = (I_{\text{max}} - I)/(I_{\text{max}} - I_{\text{min}}) \). \( I \) is the intensity at the given glucose concentration, \( I_{\text{min}} \) is the intensity at the minimum signal, and \( I_{\text{max}} \) is the intensity at the maximum signal. A sigmoidal curve was fitted to the plot of \( \alpha \) vs. log [glucose concentration] Molar using Origin software (Northampton, MA, USA). The \( K_d \), center of the dynamic range, was determined as the glucose concentration where \( \alpha \) is equal to 0.5 on the calibration curve. Furthermore, the sensitivity of the sensors was calculated from the slope of the linear region of the sigmoidal curve.

Reversibility of the Macrosensor. Macrosensors of a single optode batch were prepared by the procedure described above using 16 wells. After the optodes were hydrated, the PBS solution was removed from all wells and 200 μl of 1 M glucose in PBS was pipetted into 8 wells. The remaining 8 wells acted as controls and contained fresh glucose-free PBS. In order to track the
changes in the fluorescence response, measurements were acquired at a sampling rate of 5 minutes for 60 minutes. Then, all solutions (glucose and control) were removed and 200 μl of fresh PBS was added to all wells. Again, measurements were obtained every 5 minutes for 60 minutes. An additional cycle was performed to yield a total experiment time of 4 hours. Response was determined by the percent difference between the average intensity of the glucose and control. Figure 3.1C was generated by averaging the fluorescence intensity of the individual macrosensors for the three time points prior to a solution change. These values were then standardized and the percent change was calculated as the difference between the average standardized intensity of the glucose and control. The error was generated from the raw intensity values using the laws of error propagation.

**Effects of Interferents.** Calibration of the macrosensors was performed as described above with PBS and glucose solutions that contained either no interferent, uric acid (20 mg/dl), ascorbic acid (3 mg/dl) or acetaminophen (20 mg/dl). The glucose concentrations used in the calibration were 0 mM, 1 mM, 3 mM, 5 mM, 10 mM, 25 mM, 50 mM, 100 mM, and 1M. Response was compared by fitting a sigmoidal curve to the plot of α vs. log [glucose concentration] Molar for each interferent using Origin software.

**Nanosensor Fabrication.** The optode was allowed to dry on a glass surface for at least 4 hours. The optode film was removed and transferred to a glass vial. The vial was then charged with 5 ml of PBS and 5 mg of surface modifier, 1,2-distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethyleneglycol)-550] (PEG lipid), in 0.5 ml chloroform. This solution was sonicated with a probe-tip sonicator (Branson, Danbury, CT, USA) at 40% amplitude for 3 minutes. The residual polymer was discarded to yield the nanosensor solution.
**Particle Sizing and Zeta Potential.** Particle size and zeta potential of the nanosensors were determined using a nanosizer (Nano Series ZS90, Malvern, Worcestershire, UK). Data was acquired for nanosensors made on five different days. A minimum of five runs was performed for each fabrication. The average and standard deviation of measurements were then calculated ($n = 31$). The reported size is the Z-Average or the “cumulants mean” which is an intensity mean of the nanosensors. \(^{50}\)

**Spectrum of Nanosensors.** Data was acquired in a Spectramax Gemini EM microplate fluorometer (Molecular Devices) by exciting at 460 nm and scanning the emission from 510-690 nm. 100 µl of nanosensors in PBS was pipetted into 8 wells of a 96-well optical bottom plate. Glucose in PBS was added to each well to bring final concentrations to 0 mM, 0.5 mM, 5 mM, 15.8 mM, 50 mM, 158 mM, 0.5 M or 1 M. This process was repeated in quadruplicate for each glucose concentration with each well having equivalent volume.

**Calibration of Nanosensors.** Data was acquired in a Spectramax Gemini EM microplate fluorometer exciting at 460 nm and emitting at 570 nm with a cutoff of 515 nm. 60 µl of nanosensors in PBS was pipetted into 12 wells of a 96-well optical bottom plate. Glucose in PBS was added to each well to bring final concentrations to 0 mM, 0.5 mM, 1 mM, 1.5 mM, 2.5 mM, 4 mM, 5 mM, 12.5 mM, 25 mM, 50 mM, 250 mM, and 500 mM glucose. This process was repeated in quadruplicate for four sets of glucose nanosensors at each glucose concentration with each well having equivalent volume. This process was repeated at 4 and 8 hours after sensor fabrication in order to determine the lifetime of the sensors. Response was determined by expressing the data as $\alpha = (I_{\text{max}} - I)/(I_{\text{max}} - I_{\text{min}})$. The error for $\alpha$ was calculated from the raw fluorescence intensity values using the laws of error propagation. A sigmoidal curve was fitted to the plot of $\alpha$ vs. log [glucose concentration] Molar using Origin software.
**Leaching of Nanosensors.** Data was acquired in a Spectramax M2 fluorometer (Molecular Devices) exciting at 460 nm and emitting at 570 nm with a cutoff of 515 nm. 500 µl of nanosensors in PBS mixed with an additional 500 µL of PBS was placed in a cuvette. The intensity of the nanosensors was tracked every 10 minutes for approximately 17 hours. The intensity was normalized to its initial value in order to get the percent change over time.

**Photobleaching of Nanosensors.** Optical data was collected on an IVIS® 200 (Caliper, Hopkington, MA, USA) in vivo animal imager with both excitation and emission filters set to GFP. Three 1 µl spots of nanosensors were placed on a glass microscope slide and imaged every 2 minutes for 94 minutes. The average efficiency for each spot was standardized to the time after the spots had dried. The standardized average efficiency was tracked over time in order to determine the effects of photobleaching.

**In Vivo Studies.** Optical data was collected with the IVIS® Spectrum (Caliper, Hopkington, MA, USA) in vivo animal imager. Each mouse was shaved and then fasted for 18 hours. Four mice (two glucose and two controls) were employed in each experimental run. Mice were anesthetized with 3% isoflurane in oxygen. Blood glucose measurements from the tail of each mouse were taken with a LifeScan OneTouch® Ultra® (Langhorne, PA, USA) glucose meter and corresponding test strips prior to injection of the nanosensors. Then, 10 µl of the nanosensors were injected into the subcutaneous region at four locations along each of their backs. All four mice were simultaneously imaged every 2 minutes for 15 minutes. Excitation and emission wavelengths were chosen as 500 and 600 nm, respectively, based on the available filter sets on the IVIS® Spectrum. At this point, a second blood measurement was taken with the glucometer prior to oral gavage. Then, the two control mice were administered an oral gavage of 300 µl saline, and the two glucose mice were administered 300 µl of saline containing...
glucose (3 g/kg). Glucose administration through oral gavage has been shown in previous mice studies to produce a systemic rise in blood glucose levels$^{51}$ and was covered under the approved animal protocol. All four mice were simultaneously imaged every 2 minutes for 10 minutes. Then, another blood measurement was taken with the glucometer. The same cycle of imaging followed by blood glucose measurement was repeated until a total of 6 cycles had been performed. This process was repeated to obtain an $n = 4$ for both the control and glucose.

At the point of oral gavage, the percent change of optical intensity was set to 0%. Any loss of optical intensity detected in the control mice was determined to be diffusion of the nanosensors away from the point of injection. The optical data collected from each glucose mouse was normalized versus the control mice signal in order to accurately estimate the percent change attributable to glucose and account for the effect of diffusion on signal loss. A plot of the percent optical change and blood glucose measurements versus time for each mouse is shown in Figure 3.6.

In order to correlate the percent optical change from the nanosensors with blood glucose measurements, the average percent intensity change was determined for each blood glucose reading by averaging the two optical intensities closest to the reading. The percent optical change before and after blood readings was averaged for measurements obtained during the experiment. The initial and end blood measurements were correlated to the two points after and before blood readings, respectively. A line was fitted to the xy-scatter plot of percent optical change data points vs. glucose concentration for each mouse using Origin software. The fitted line provided a calibration between the optical response of the nanosensors and the blood glucose concentration for each mouse. In order to compare the calibrations among the experiments, the glucose concentration for each optical data point was determined from the equation of the fitted
line for each mouse. An xy-scatter plot of the glucose concentration from the optical data vs. measured blood glucose readings for all mice is shown in Figure 3.3C. A line was fitted to this plot using Origin software. The correlation coefficient from this plot describes the variability among these data points and the four calibrations.

3.4 Results And Discussion

Macrosensor Development. The development of the glucose-sensitive nanosensors first begins on the macro-scale so that the optimal sensor formulation is determined prior to nanosensor fabrication. The sensor matrix consists of plasticized poly(vinylchloride) carboxylate, octylboronic acid, alizarin and the ionic additive, tridodecylmethylammonium chloride. The sensor components are dissolved in THF creating an optode. Through optimization of these molecular components and their respective ratios, an optical macrosensor was developed to respond to glucose in the physiological range (1-25 mM). 52 As depicted in Figure 3.1A, the fluorescence response of the macrosensor was measured from low (1 mM) to high (1 M) levels of glucose. The dissociation constant (K_d) for the macrosensor was determined to be 19 mM by sigmoidal curve fit analysis. The linear region of the curve encompasses the physiological range where the sensors have a sensitivity of 0.2 mM per percent change of fluorescence intensity. We also measured macrosensor response to glucose in the presence of common interferents of electrochemical reactions that produce falsely high readings for numerous enzyme-based sensors. 49 These compounds, uric acid, ascorbic acid and acetaminophen did not result in statistically significant differences in the response (Figure 3.1B). Boronic acids are known to bind to diols such as nucleotides and saccharides, 36,46,53 but the interference of other sugars such as fructose and galactose was not measured because of their low concentration (<1 mM) 54 found
Figure 3.2. Response of optical macrosensor to glucose. (A) Calibration curve and (B) effects of chemical interferents on the calibration. Mean ± SD (n = 24) for A and mean ± SD (n_{control} = 9, n_{ascorbic acid} = 8, n_{uric acid} = 9, n_{acetaminophen} = 8) for B is shown. There was no statistically significant difference in response to these common interferents with p > 0.01 when compared to the control at each concentration. (C) Reversibility of the response to glucose. Percent change refers to the loss of fluorescence intensity exhibited by macrosensor when exposed to 1M glucose in PBS versus control (PBS) (n_{control} = 7 and n_{glucose} = 6).
at physiological conditions which is below the dynamic range of our sensor formulation. Furthermore, we assessed the macrosensor response from pH 6.8 to 7.8 due to its physiological relevance (blood pH=7.45) and found no statistical difference in response within this range (p > 0.05, data not shown). The macrosensor response would be expected to shift at more acidic and basic pHs due to alizarin’s pKas at 6.2 and 11.1; however, this pH range was not analyzed since these values fall outside the relevant physiological range. In addition, the response of the macrosensor to glucose proved to be reversible (Figure 3.1C). This cycle of response and recovery could be repeated multiple times with only slight loss of response. Due to the size of the macrosensor, the response was measured over one hour. This response time is approximately 3 times longer than sodium macrosensors (data not shown). Miniaturizing the sodium sensors to the nanoscale reduces the estimated response time to microseconds. Thus miniaturizing the glucose macrosensors to the nanoscale should result in a response time on the order of at least seconds which would be acceptable for a monitoring device.

**Nanosensor Development.** Nanosensors were constructed through rigorous sonication of a mixture containing dried optode, PBS, and PEG-lipid surface modifier dissolved in chloroform. The modifier is an amphiphilic molecule that readily inserts into the hydrophobic sensor, leaving the hydrophilic, biocompatible component on the outside of the nanosensor exposed to the aqueous environment. This coating stabilizes the nanosensors in solution and imparts biocompatibility. The average size of the nanosensors was 74.2 nm ± 39.8 nm, as measured with a Nano Series ZS90 particle sizer (Malvern, Worcestershire, UK).

The response of the nanosensors to glucose was initially examined in a well plate, and a wide dynamic range was observed (Figure 3.2). As can be seen, even at high concentrations of glucose, saturation of the signal was not possible. The sensors had a measured $K_d$ and sensitivity
of 38 mM and 2 mM, respectively. The nanosensors still responded within the physiological range, but suffered some loss of sensitivity due to the miniaturization process. The response of the nanosensors to glucose was also monitored over time. Minimum differences were observed between the 0 hour and 4 hour calibrations, but a slight shift in nanosensor response was seen after 8 hours (Figure 3.2). The slight shift in response could be due to the gradual leaching of sensor components caused by the increased surface area to volume ratio of the sensors.

**In Vivo Response of Nanosensors.** The glucose-sensitive nanosensors were evaluated *in vivo* by tracking the qualitative changes in blood glucose levels, measured with a glucometer, against the optical response of the nanosensors. Mice were anesthetized then injected subcutaneously with nanosensors in PBS along the back (Figure 3.3A). Images were taken for 15 minutes to establish a baseline fluorescence intensity. During this time, a decrease in fluorescence intensity was observed, which was attributed to the equilibration of the sensors with the resting glucose levels of the mice and sensor diffusion away from the injection site. Though we could not quantifiably monitor fluorescence staining due to the optical resolution of the instrument, we believe that the sensors diffused into the blood stream. To account for sensor diffusion away from the point of injection, all mice were paired with controls (no oral glucose). Blood glucose measurements were taken from the tail vein and analyzed with a LifeScan OneTouch® Ultra® glucometer. The LifeScan OneTouch® Ultra® glucometer has been FDA-approved for measuring glucose levels in diabetic care. After baseline was established, mice were given an oral gavage of either saline (control) or glucose solution. This method of administering glucose has been shown effective for increasing blood glucose levels in mice. Optical measurements were subsequently taken every 2 minutes for a 10 minute span followed by a blood glucose reading. This cycle of optical and blood measurements was conducted for one hour after glucose gavage.
Figure 3.3. Response of optical nanosensors to glucose in PBS. Calibration curve of the nanosensors at 0, 4, and 8 hours. Average of quadruplicate samples from four independent samples.
Figure 3.4. *In vivo* response of nanosensors to glucose. (A) Image of mouse injected at four locations with glucose-sensitive nanosensors. Image was obtained with an IVIS®-Spectrum imaging system. Excitation and emission wavelengths were 500 nm and 600 nm, respectively. Intensity bar displays the normalized fluorescence efficiency, which represents the fractional ratio of fluorescent emitted photons per incident excitation photon. Residual background fluorescence was attributed to remaining fur. (B) The representative response to oral gavage of the blood glucose (red) and fluorescence of the glucose nanosensors (black). Mean ± SD for one mouse is shown. (C) Correlation *in vivo* between optical response of nanosensors and blood measurements. Glucose concentration from optical data vs. blood glucose is shown for all mice (n = 4). Error bars are omitted for clarity. A line was fitted to the plot (slope = 1.0, $R^2 = 0.75$).
Figure 3.3B displays the optical response and blood measurements for an individual mouse over the course of an experiment. The sensor response correlated with blood glucose readings, and this relationship was maintained over the course of analysis. Both monitoring methods initially detected a significant increase in glycemic levels due to the glucose gavage. The glucose levels eventually stabilized, presumably as the mouse produced an endogenous insulin response to the elevated glucose levels. The nanosensors demonstrated a rapid response to variations in glycemic levels, which is an inherent feature of the chemical equilibrium established in our sensor design. This attribute of the nanosensors allowed for a more continuous glucose analysis than the single-point measurements. The nanosensors tracked blood glucose concentrations from 66 mg/dl, 3.7 mM, to 427 mg/dl, 23.7 mM. The large error bars for the optical response could be due to the injection technique and biological variation such as the metabolic rate for each mouse. No error bars are present for the blood glucose measurements as only one blood sample was taken each sequence because of blood volume limitations in mice.58 The correlation between optical response and blood measurements was consistent for each mouse examined in the study (Figure 3.6). Although the upper detection limit of the blood glucose monitor is 600 mg/dl, higher glycemic levels are capable of being optically detected as shown in Figure 3.2.

The in vivo measurements for all mice were correlated to confirm the relationship between optical response and blood glucose concentration in each experiment. The average percent intensity change was determined for each blood glucose reading. For each mouse, a linear relationship between optical and blood glucose was obtained (data not shown). The calibration for each mouse was then used to determine the calculated glucose concentration from the optical data at the time of each blood measurement. Figure 3.3C displays the xy-scatter plot
Figure 3.5. (A) Emission spectrum of alizarin at various glucose concentrations ($n=4$ for each concentration). (B) Leaching of the glucose-sensitive nanosensors over approximately 17 hours. (C) Photobleaching of the glucose-sensitive nanosensors in the IVIS 200 Biophotonic Imager. Mean ± SD ($n=3$) is shown.
Figure 3.6. The response to oral gavage of the blood glucose (red) and fluorescence of the glucose nanosensors (black) for all mice. Mean ± SD for each mouse is shown.
of these data points for all mice. The linear fit of the data indicates that optical measurements correlated to blood glucose measurements over the entire range of glucose concentrations when the data from all animals is combined. Throughout the in vivo experiments, glycemic levels were found to vary between 66 mg/dl, 3.7 mM, and 537 mg/dl, 29.8 mM. The glucose concentrations obtained from the optical response of the glucose-sensitive nanosensors were consistent with the measurements acquired from the glucometer in this critical span. This correlation demonstrates that subcutaneous injections can reflect blood glucose levels and that nanosensor response is unaffected by the in vivo conditions. Literature is available regarding regional differences and time lags in glucose levels throughout the body; however, accounting for these differences will be the focus of future work. In addition, the change in optical signal is not significantly attenuated by the skin of the mouse. Future work will include optimizing the sensor response within the hypoglycemic range, performing in vivo sensor calibration, and incorporating a reference dye into the sensors to minimize variation in sensor response due to injection techniques and inherent biological variation.

3.5 Conclusion

In summary, we have designed glucose-sensitive nanosensors that exploit the competitive binding properties of aryl and alkyl diols with boronic acids. This chemistry is maintained within a hydrophobic core which limits effects of interferents and maintains, in close proximity, the components required for reversibility of the competitive binding. The nanosensors have not only demonstrated this reversible response to dynamic changes in glucose concentration, but they also have a dynamic range encompassing physiologically relevant glucose levels. Furthermore, the nanosensors tracked rapidly changing glucose concentrations in vivo which
corresponded to blood glucose levels measured with a glucometer. The ability of the nanosensor system to monitor glucose dynamics in vivo could be applied to diabetes treatment as well as applications in research, such as monitoring the effects of β-cell function or novel treatments for the disease.


3.6 Acknowledgements

This work was supported by the National Institute of General Medicine of the National Institutes of Health under award number R01 GM084366 and by Internal Research and Development funding from The Charles Stark Draper Laboratory.

3.7 Supplementary Information

The Supplementary Information includes a calibration spectrum, leaching measurements, and photobleaching measurements for the glucose-sensitive nanosensors. The optical response of the glucose-sensitive nanosensors and blood glucose measurements over time for all mice in the in vivo experiments are also included.
Chapter 4: Gel Encapsulation of Glucose Nanosensors for Prolonged *In Vivo* Lifetime

4.1 Chapter Overview

*Background:* Fluorescent glucose-sensitive nanosensors have previously been used *in vivo* to track glucose concentration changes in interstitial fluid. However, this technology was limited because loss of fluorescence intensity due to particle diffusion from the site of injection. In this study, we encapsulated the nanosensors into injectable gels to mitigate nanosensor migration *in vivo*.

*Methods:* Glucose-sensitive nanosensors were encapsulated in two different commercially available gelling agents: Gel 1 and Gel 2. Multiple formulations of each gel were assessed *in vitro* for their nanosensor encapsulation efficiency, permeability to glucose, and nanosensor retention over time. The optimal formulation for each gel, as determined from the *in vitro* assessment, was then tested in mice and the lifetime of the encapsulated nanosensors was compared to controls of nanosensors without gel.

*Results:* Five gel formulations had encapsulation efficiencies of the nanosensors greater than 90%. Additionally, they retained up to 20% and 40% of the nanosensors over 24 hours for Gel 1 and Gel 2, respectively. *In vivo,* both gels prevented diffusion of glucose nanosensors at least three times greater than the controls.

*Conclusions:* Encapsulating glucose nanosensors in two injectable gels prolonged nanosensor lifetime *in vivo*; however, the lifetime must still be increased further to be applicable for diabetes monitoring.
4.2 Introduction

The worldwide prevalence of diabetes has spurred the interest in continuous glucose monitoring systems as an alternative to the finger-prick method. Glucose monitors such as DexCom™ STS™ Continuous Glucose Monitoring System and Medtronic’s Guardian® REAL-Time Continuous Glucose Monitoring System are commercially available and FDA approved to track trends in glucose levels. However, further research into novel approaches for glucose monitoring is still of interest in order to prolong sensor lifetimes, improve accuracy, and minimize invasiveness of measurements. Several reviews such as those written by Wang, Pickup, Steiner, and Cash cover the scope of these developments that include the extension of nanotechnology to glucose sensing. For example, glucose micro and nanosensors provide the benefits of rapid response times and ease of implantation due to their large surface area to volume ratio and small size. McShane and colleagues are developing a “smart tattoo” composed of dissolved-core alginate microspheres to be implanted into the skin and monitor glucose levels in the interstitial fluid. These sensors use fluorescence resonance energy transfer (FRET) and a competitive binding mechanism with a non-catalytic mutant of glucose oxidase to repeatably monitor reversible changes in glucose at physiological levels. In other work, nanosensors using boronic acids as a non-biological recognition element for glucose have also been investigated because of boronic acids’ affinity for 1,2 diols. A common sensing mechanism involving boronic acids is the competitive binding between a fluorescent reporter and glucose for the boronic acid binding site. The fluorescence of the reporter is different in the bound and unbound states, yielding a change in measured signal as the reporter is displaced from the boronic acid. Wang et al. developed glucose sensing vesicles using this mechanism with phenylboronic acid, glucose and the fluorescent reporter, alizarin red S (ARS). ARS was
electrostatically coupled to cationic quaternary ammonium salts that self-assembled into vesicles in solution. As glucose was added to the system, ARS was displaced by glucose which resulted in a decrease in intensity because the unbound ARS is significantly less fluorescent. Our group utilizes a similar competitive binding scheme as the basis for functional nanosensors, but the sensing components are embedded in a lipophilic, highly-plasticized polymeric particle into which glucose is extracted. The hydrophobic particle design has several advantages that include isolation of the sensing components from biological fluid to prevent biofouling and tunability of the system to adjust the dynamic range.

Our glucose-sensitive nanosensors successfully tracked changes in glucose levels when injected subcutaneously along the backs of mice. However, the monitoring time was limited to 1 hour due to loss of signal intensity at the injection site. Studies conducted by Gopee with intradermally-injected quantum dots found that quantum dots migrated from the site of injection with 60% of quantum dots remaining at the injection site after 24 hours. The nanosensors were implanted similarly in the skin and migration was assumed to be the main cause of signal loss over time. To mitigate sensor migration in our system, sensor geometry was altered into microworms that limited sensor diffusion and prolonged their lifetime in the skin more than the nanosensors alone. However, the yield of microworms was not sufficient for in vivo monitoring. In this study, we encapsulated glucose nanosensors in injectable gels as another approach to prevent nanosensor diffusion in vivo. Injectable gels have made an impact on fields such as drug delivery and tissue engineering because they form under mild conditions, are easily implantable, and biodegradable. These advantages make injectable gels ideal implantation vehicles for the glucose nanosensors. The primary focus of this study was to investigate injectable gels for limiting nanosensor migration in vivo. Important characteristics of the glucose
sensors such as dynamic range, sensitivity, reversibility, and lifetime have been previously addressed\(^{10,69}\) and no work on sensor development is discussed here.

### 4.3 Materials and Methods

**Materials.** Matrigel™ basement membrane matrix (Gel 1) (growth factor reduced, phenol red-free, LDEV-free) and 31 gauge insulin syringes were purchased from BD Biosciences (Franklin Lakes, NJ). Extracel-X® hydrogel kit (Gel 2) was purchased from Glycosan Biosystems Inc. (Alameda, CA). Bis(2-ethylhexyl) sebacate (DOS), tridodecylmethylammonium chloride (TDMAC), alizarin (dye content 97%), tetrahydrofuran (anhydrous, \(\geq 99.9\%\), inhibitor free) (THF), and chloroform (Chromasolv®, \(\geq 99.8\%\)) were purchased from Sigma-Aldrich (St. Louis, MO). Octylboronic acid was purchased from Synthonix, Inc. (Wake Forest, NC). Gibco® Phosphate buffered saline (PBS) (1x, pH=7.4) was acquired from Life Technologies (Grand Island, NY) and poly(vinyl chloride) carboxylated (PVC-COOH) was purchased from Scientific Polymer Products, Inc. (Ontario, NY). Amicon Ultra centrifugal filters (molecular weight cut-off, 100 kDa) were obtained from Millipore (Billerica, MA). 1,2-distearoyl-\(sn\)-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-550] (ammonium salt) in chloroform (PEG 550) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). SKH1-E mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA).

**Optode cocktail.** Macro and nanosensors were fabricated from an optode cocktail that contained all sensing components: 30 mg of PVC-COOH, 60 \(\mu\)L of DOS, 3 mg of octylboronic acid, 4 mg TDMAC, and 1 mg of alizarin all dissolved in 500 \(\mu\)L of THF. The selection, optimization, and characterization of these components for glucose sensing are described elsewhere.\(^{10,69}\)
Nanosensor fabrication. Fabrication of glucose nanosensors have been described previously. Briefly, the optode cocktail was dried for at least 4 hours on a glass plate. It was then removed from the plate and placed into a scintillation vial along with 5ml of PBS, 5mg of PEG-550, and 500 µL of chloroform. The mixture was sonicated using a Branson digital sonifier (Danbury, CT) for 3 minutes at 40% amplitude. The nanosensors were concentrated using Amicon Ultra centrifugal filters prior to encapsulation in the gelling agents.

Gel preparation. Two commercially available gels were selected for encapsulating the nanosensors. Each gel has applications in cell encapsulation and tumor growth models. Gels were prepared according to the manufacturer’s instructions and diluted with nanosensors and PBS according to the ratios in Tables 4.1 and 4.2 to a total volume of 300 µL. The gelling agents were allowed to gel in a 31 gauge insulin syringe for at least 20 minutes and 90 minutes for Gel 1 and Gel 2, respectively. All gels were formed prior to the beginning of experiments. Gel 1 is formed by simply bringing the gel to room temperature. Gel 2 is formed by crosslinking a thiol-modified hyaluronan and thiol-modified gelatin with a thiol-reactive crosslinker, polyethylene glycol diacrylate. For all the gel formulations listed in Table 4.2, hyaluronan and gelatin were used in equal parts and polyethylene glycol diacrylate was 20% of the total gel volume. In the case of both gels, dilution of the gel components with the nanosensors and PBS decreases their stiffness. To serve as a control for no gel, nanosensors were diluted with only PBS to a final volume of 300 µL.

Nanosensor encapsulation efficiency. 100 µL of prepared gel with sensors or the no gel control was injected into a 96-well optical bottom well plate. Fluorescence measurements were acquired on a SpectraMax Gemini EM plate reader (Molecular Devices, Sunnyvale, CA) at 460 nm and 570 nm for excitation and emission wavelengths, respectively. After the initial reading, the gels
Table 4.1. Ratio of components for each Gel 1 formulation. All gels were made with a total volume of 300 µL.

<table>
<thead>
<tr>
<th>Gel 1: Nanosensors/PBS</th>
<th>Nanosensors</th>
<th>PBS</th>
<th>Gel 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5:1</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2:1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1:1</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
### Table 4.2. Ratio of components for each Gel 2 formulation. All gels were made with a total volume of 300 µL.

<table>
<thead>
<tr>
<th>Gel 2: Nanosensors/PBS</th>
<th>Nanosensors</th>
<th>PBS</th>
<th>Gel 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5:1</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>3:1</td>
<td>1</td>
<td>0.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2:1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>
were washed with 100 µL of PBS, the PBS was removed, and then a second measurement was acquired. The encapsulation efficiency was calculated as the fluorescence intensity of the after wash measurement divided by the initial measurement for each sample. This ratio was then expressed as a percentage.

**Glucose permeability through gels.** To test glucose permeability, glucose macrosensors were used because they can be adhered to a surface eliminating the possibility of unencapsulated nanosensors contributing to sensor response. The macrosensors are formed by pipetting the optode cocktail (2 µL) onto glass discs adhered to the bottom of a 96-well optical bottom well plate and then dried. 200 µL of PBS was added to each well and the sensors were hydrated in PBS for at least 4 hours until their fluorescence intensity stabilized. All fluorescence measurements were acquired at 460 nm and 570 nm for excitation and emission wavelengths, respectively using a SpectraMax Gemini EM plate reader. After hydration, 100 µL of gels were injected over the macrosensors and adjusted until they covered the entire bottom of the well plate. Gels were prepared according to the ratios in Tables 4.1 and 4.2, but the nanosensors were substituted with PBS. For a no gel control, 100 µL of PBS was substituted for the gels. The concentration of glucose added was dependent upon the gel used and its ratio of dilution such that for all cases the final glucose concentration in each well was equal to 9,000 mg/dl. The macrosensors and nanosensors have a center of dynamic range of 342 mg/dl and 684 mg/dl, respectively, but high concentrations of glucose were used here in order to maximize sensor response. Gel 1 is provided in 0.5 mg/dl of glucose Dulbecco’s Modified Eagle Medium and the glucose concentration in each Gel 1 formulation was calculated. Gel 2, as provided by the manufacturer, is reconstituted in PBS and we assumed that 100 µL of Gel 2 was equivalent to 100 µL of PBS. The fluorescence response of the macrosensors was monitored for 3 hours at 5
minute intervals. Fluorescence measurements were normalized to time 0 and then subtracted from the control wells for each gel formulation. This difference was then expressed as a percent change and the error bars were calculated using error propagation.

**Gel retention of nanosensors.** Glucose nanosensors were encapsulated in gels as described in the gel preparation section. 40 µL of gel was injected onto a glass bottom petri dish that was then filled with 4 ml of PBS. The gels were placed in an incubator at 37°C for ten minutes and then imaged using an IVIS Lumina II small animal imager (Caliper Life Sciences, Hopkinton, MA). Images were acquired with 465 nm excitation and 580 nm emission filters. They were recorded approximately every half hour for the first two hours and then at 6.5 hours and approximately 24 hours. Between measurements, the gels were stored in an incubator at 37°C. The bulk degradation of the nanosensors (sensor degradation, component leaching, photobleaching) over this time was also monitored to separate these effects from the loss of intensity caused by diffusion. For the bulk degradation controls, 40 µL of glucose nanosensors in PBS was placed in microcentrifuge tubes and imaged at the same time points as mentioned above. In between measurements, the nanosensors were stored in an incubator at 37°C. For analysis, the total radiant efficiency from a region of interest encompassing either the gel or the nanosensors for the bulk degradation control was selected, normalized to the time 0 measurement, and plotted over time to determine the rate of nanosensor diffusion out of the gel.

**In vivo testing.** All animal procedures were approved by Northeastern University’s Institutional Animal Care and Use Committee. Glucose nanosensors were encapsulated in gels according to the protocol in the gel preparation section or diluted with PBS for the no gel control. SKH1-E mice were anesthetized and 40 µL of each gel with nanosensors and a no gel control were injected intradermally along their backs. Intradermal injections were achieved by pinching the
skin and then injecting nanosensors with or without gel while the syringe was inserted parallel to
the skin. Characteristic of intradermal injections, a bleb or small skin welt was visible after
injection.76 Mice were imaged with an IVIS Lumina II small animal imager with excitation and
emission filters at 465 nm and 580 nm, respectively. Images were acquired for the first 60
minutes at 10 minute intervals and an additional measurement was taken 3 hours post-injection.
Similar to the gel retention experiments, the total radiant efficiency from a region of interest
surrounding each injection spot was selected for analysis. An additional region of interest with
skin only was also selected to determine the background skin fluorescence. The background
fluorescence from the skin was subtracted from the region of interest and the data was
normalized to time point 0.

4.4 Results

**In vitro characterization.** Both gelling systems were assessed for three critical characteristics:
encapsulation efficiency, glucose permeability into the gel, and sensor retention over time. Gel
formulations were down selected based on these *in vitro* characteristics prior to *in vivo* testing.
First, nanosensors were mixed with Gel 1 and Gel 2 and diluted at different ratios to adjust gel
stiffness. Their encapsulation efficiency of nanosensors is shown in Figure 4.1. All gels
encapsulated the nanosensors better than the control and five out of six gels loss only 10%
percent of their intensity after washing. Gel 1 showed no correlation between stiffness and
encapsulation efficiency whereas the encapsulation efficiency of Gel 2 decreased with decreased
stiffness. The encapsulation efficiency of Gel 2, 2:1, was not down-selected in further
experiments due to its poor performance compared to the other formulations.
Figure 4.1. Encapsulation efficiency of gels. Glucose nanosensors were mixed with gelling agents with variable stiffness and allowed to gel. Shown here is the percent change in fluorescence intensity after the gels were washed with PBS from their initial fluorescence. $n$ is equal to 5 for the control, 2:1 Gel 1, and 2:1 Gel 2 and $n$ is equal to 6 for 5:1 Gel 1, 1:1 Gel 1, 5:1 Gel 2, 3:1 Gel 2. Error bars represent standard deviations.
Gel encapsulation of the nanosensors may affect their ability to monitor real-time changes in glucose concentration because of limited to no glucose permeability into the gel and potential delays in response. Therefore glucose permeability into the gels was tested. To determine glucose permeability, glucose macrosensors were used rather than nanosensors to eliminate unencapsulated nanosensors from contributing to the fluorescence response to glucose. Additionally, glucose macrosensors respond over the course of 1 hour\textsuperscript{10} and thus added to the overall monitoring time required. The percent change in signal of the glucose macrosensors as a function of time are shown in Figure 4.2 for Gel 1 and Figure 4.3 for Gel 2. The gels were permeable to glucose with the response kinetics linear as a function of time until maximum response was achieved. The gel coating caused a delay in sensor response because glucose must diffuse through the entire gel layer before being sensed by the glucose macrosensors. Previous research on glucose diffusion through gels calculate these delays using lag-time analysis where the delay is the x-intercept from a linear fit of total glucose diffusion through the gel membrane over time.\textsuperscript{77-79} In the case of our sensors, fluorescence response is related to glucose concentration and therefore the x-intercept of the linear fluorescence response was used for delay calculations. For Gel 1, the gel coating caused a lag time for initiating sensor response by 0, 16, and 19 minutes for the ratios 5:1, 2:1, and 1:1, respectively. Similarly, for Gel 2 the response was delayed by 44 and 52 minutes for the ratios of 5:1 and 3:1, respectively. For both Gel 1 and Gel 2, variability in sensor response increased with gel stiffness, but all gels in each grouping had a similar percent change to glucose and therefore, no down selection was made after this stage. Finally, the assessment of nanosensor-retention in the gels was performed. Figure 4.4A-C shows the Gel 1 ratios with encapsulated nanosensors at 4 distinct time points. Over 24 hours, the fluorescence intensity of the immobilized sensors decreased as the total number of sensors
Figure 4.2. Glucose permeability through Gel 1. Glucose macrosensors were layered with different Gel 1 formulations of variable stiffness and then exposed to 0 g/dl and 9,000 g/dl of glucose for the control and experimental group, respectively. The percent difference between the controls and experimental groups are plotted against time for no gel (—■—), 5:1 (Gel 1: PBS, --○--), 2:1 (Gel 1: PBS, ⋯▲⋯), and 1:1 (Gel 1: PBS, --▼--). n is equal to 6 for the no gel control. n is equal to 5 for formulation 5:1 and formulation 2:1. n is equal to 5 and 4 for the 1:1 formulation experimental and control groups, respectively. Error bars were calculated using error propagation.
Figure 4.3. **Glucose permeability through Gel 2.** Glucose macrosensors were layered with different Gel 2 formulations of variable stiffness and then exposed to 0 g/dl and 9,000 g/dl of glucose for the control and experimental group, respectively. The percent difference between the controls and experimental groups are plotted against time for no gel (— ■ —), 5:1 (Gel 2: PBS, --○--), and 3:1 (Gel 2: PBS, ⋯ ▲ ⋯). n is equal to 6 and 4 for the no gel control and Gel 2, respectively. Error bars were calculated using error propagation.
decreased. Sensors diffused away from the edges of the gel, but were still retained in the center, as anticipated. Figure 4.4D plots the average total radiant efficiency for each gel ratio over time along with the bulk degradation control. Since bulk degradation of the sensors encompasses sensor degradation, component leaching, and photobleaching, the difference of intensities between the bulk degradation control and gels is assumed to be diffusion of sensors out of the gel. To determine differences in the rate of signal loss between the gels, the data between 0 and 6.5h was fitted to the exponential model:

\[ y = Ae^{R_0t} \]

where \( A \) is the initial value, \( R_0 \) is the rate of decay, and \( t \) is time. The decay rates are displayed in Table 4.3. All three ratios of Gel 1 had similar decay rates. Correspondingly, Figure 4.5 shows the fluorescence decay of Gel 2 ratios with encapsulated nanosensors over time. The total change in fluorescence signal and decay rates are less than Gel 1 at the various ratios (Table 4.3). In the case of both gels, each formulation performed similarly except for their delayed response in the glucose permeability experiments. Therefore, 5:1 Gel 1 and 5:1 Gel 2 were selected for the \textit{in vivo} experiments because they had the shortest delays in responding to glucose.

\textbf{In vivo demonstration.} \textit{In vitro} characterization of the gels demonstrated that gels can be used as an encapsulation vehicle for the glucose nanosensors \textit{in vivo}. Mice were intradermally injected with 5:1 Gel 1, 5:1 Gel 2, and a no gel control in three separate spots along the back (Figure 4.6A). Over the course of 1 hour, the average normalized total radiant efficiency for 3 mice decreased to 14%, 41%, and 55% of the initial value for no gel, Gel 1, and Gel 2, respectively. For the first hour, the difference between the 2 gels and the control was significant \((p < 0.05)\); however, there was no significant difference between Gel 1 and Gel 2 \((p > 0.05)\). By
Figure 4.4. Gel 1 retention of glucose nanosensors. Fluorescent images of Gel 1 with glucose nanosensors over time for the (A) 5:1 and (B) 2:1 and (C) 1:1 formulations of Gel 1. (D) Normalized radiant efficiency of the gels over time for the bulk degradation control (—•—), 5:1 (—○—), 2:1 (⋯▲⋯), and 1:1 (⋯▼⋯) formulations. n is equal to 3 for all samples and error bars represent standard deviations.
**Table 4.3.** Decay rates of the average total radiant efficiency over 6.5 hours for the bulk degradation control and each formulation of Gel 1 and Gel 2.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Decay Rate (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk Degradation Control</td>
<td>-0.02±0.01</td>
</tr>
<tr>
<td>5:1 Gel 1</td>
<td>-0.14±0.03</td>
</tr>
<tr>
<td>2:1 Gel 1</td>
<td>-0.12±0.04</td>
</tr>
<tr>
<td>1:1 Gel 1</td>
<td>-0.14±0.02</td>
</tr>
<tr>
<td>5:1 Gel 2</td>
<td>-0.08±0.01</td>
</tr>
<tr>
<td>3:1 Gel 2</td>
<td>-0.08±0.01</td>
</tr>
</tbody>
</table>
Figure 4.5. Gel 2 retention of glucose nanosensors. Fluorescent images of Gel 2 with glucose nanosensors over time for the (A) 5:1 and (B) 3:1 formulations of Gel 2. (C) Normalized radiant efficiency of the gels over time for the bulk degradation control (---), 5:1 (--○--), and 3:1 (···▲···) formulations. n is equal to 3 for all samples and error bars represent standard deviations.
three hours, all three conditions had decreased to below 20% of the initial normalized efficiency and there was no significant difference between all three conditions. The fluorescence of the sensors was completely diminished by 24 hours (data not shown).

4.5 Discussion

Gel 1 and Gel 2 were selected for these studies because they can be easily modified for stiffness through dilution\textsuperscript{74,75} and they are amenable to nanosensor incorporation during the gelling process. Three parameters were used to assess and down select the optimal formulations for \textit{in vivo} testing: encapsulation efficiency of the nanosensors, glucose permeability through the gel, and nanosensor retention within the gel over time.

The particles being suspended had the potential to inhibit gelation, as was noted with other matrices (data not shown). At several different formulations for Gel 1 and Gel 2, incorporating a sufficiently high concentration of nanosensors for \textit{in vivo} detection into the pre-gelled components did not inhibit gelation. Additionally, since it is critical that implanted nanosensors can sense real-time changes in physiological concentration of glucose even while in a gel, glucose permeability through the gel was assessed. Solute transport through hydrogels is governed by polymer chain mobility, charge groups, and solute versus pore size of the gel.\textsuperscript{80} Gel 1 and Gel 2 were permeable to glucose and the shape of the kinetics of the fluorescence response of all the gels were similar to those reported by other groups on the transport of glucose through gels\textsuperscript{77-79} such as calcium alginate.\textsuperscript{77,78} This transport, as modeled using lag time analysis, has an initial lag time followed by a linear increase in the amount of glucose that has passed through the gel. In our case, the lag time for glucose diffusion through the entire gel can overshadow one of
Figure 4.6. *In vivo* testing of nanosensors encapsulated in Gel 1 and Gel 2. (A) Fluorescent images of glucose nanosensors encapsulated in Gel 1 and Gel 2 along with a no gel control over 3 hours. (B) Normalized radiant efficiency of the gels over time for the no gel control (—■—), Gel 1 (--○--), and Gel 2 (···▲···). n is equal to 3 for all samples and error bars represent standard deviations.
the main advantages of using nanosensors: their fast response. The delays in response may be shortened by reducing the amount of gel to minimize the transport time to the center of the gel. However, these transport delays may be a major disadvantage for the use of a nanosensor/gel system to monitor real-time changes in glucose. Additionally, glucose gradients that may form throughout the gel could affect sensor measurements. Therefore, we are currently investigating the use of new sensing geometries that will increase the overall size of the sensors while retaining fast sensor response times and glucose transport.

Lastly, the diffusion of the nanosensors out of the gels was investigated to determine efficacy of sensor retention over time. The average size of the glucose nanosensors is 74 nm\(^1\) and gel pore sizes less than this would be desirable for greater retention. Reported values of pore size for Gel 1 range from 26 nm to 2 µm for 1:1 dilution\(^{75,81}\) with pore size varying with Gel 1 concentration.\(^{75}\) Drastic changes in pore size of Gel 1 was not evident from our results since all Gel 1 formulations performed similarly and we predict that the average pore size for these matrices was greater than the size of the nanosensors. On the other hand, Gel 2 has been shown to retain proteins as small as 70 kDa.\(^{74}\) The average pore size for Gel 2 may be smaller than Gel 1 and thus explains the greater retention of nanosensors over time.

Previous \textit{in vivo} studies were limited to approximately an hour because of loss of signal intensity at the injection site.\(^{10}\) The majority of the signal loss was attributed to sensor diffusion from the point of injection. In our current work, immobilizing nanosensors in a gel matrix improved the lifetime to over an hour \textit{in vivo}; however, there was still a loss of signal over this time frame. Other factors such as sensor performance, photobleaching, and gel degradation contribute to loss of signal as well and inclusion of a reference dye or internal standard into the sensors will help to normalize the loss of fluorescent signal to account for these effects on
glucose measurements. Ultimately, the goal is a sensor that performs for at least one week in vivo and this will require further improvements to the nanosensors and the injection techniques, in addition to the use of gels.

4.6 Conclusion

Two commercially-available injectable gels sufficiently encapsulated glucose nanosensors while maintaining glucose permeability. Both gels prolonged sensor lifetime in vivo longer than nanosensors alone. However, more work will have to be done to improve nanosensor lifetime in order for them to be applicable for long-term diabetes monitoring.


4.7 Acknowledgements

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Chapter 5: Biodegradable Optode-Based Nanosensors for In Vivo Monitoring

5.1 Chapter Overview

Optode-based fluorescent nanosensors are being developed for monitoring important diseased states such as hyponatremia and diabetes. However, traditional optode-based sensors are composed of non-biodegradable polymers such as polyvinyl chloride (PVC) raising toxicity concerns for long-term in vivo use. Here, we report the development of the first biodegradable optode-based nanosensors that maintain sensing characteristics identical to traditional optode sensors. The polymer matrix of these sensors is composed of polycaprolactone (PCL) and a citric acid ester plasticizer. The PCL-based nanosensors yielded a dynamic and reversible response to sodium, were tuned to respond to extracellular sodium concentrations, and had a lifetime of at least 14 days at physiological temperature. When in the presence of lipase, the nanosensors degraded within 4 hours at lipase concentrations found in the liver but were present after 3 days at lipase concentrations found in serum. This development of biodegradable nanosensors is not only necessary for future in vivo applications, but it has also created a new sensor platform that can be extended to other sensing mechanisms such as for small molecules or enzymes.

5.2 Introduction

Optode sensors, the optical counterpart to ion-selective electrodes, have been developed to measure a range of ions such as sodium, potassium, and calcium and small molecules such as glucose. Miniaturization of optode sensors into micro and nanosensors has further improved their spatial resolution and response time to changes in analyte
For example, nanosensors have measured the rate of calcium release during the mitochondrial permeability transition\textsuperscript{29} and detected sodium sparks from ion channel clusters in cardiac myocytes.\textsuperscript{11} Recently, applications for optode-based sensors have extended from intracellular measurements to longitudinal \textit{in vivo} monitoring for diseased states such as hyponatremia and diabetes.\textsuperscript{10,90-92} For example, the Gratzl group has developed sliver sensors for monitoring analytes such as glucose in the skin.\textsuperscript{91,92} The clinical monitoring system proposed by our group involves injecting nanosensors into the upper layers of the skin, similar to a tattoo, and then monitoring changes in the fluorescence of the sensors using a handheld optical reader. In initial animal studies, subcutaneously-injected nanosensors tracked changes in extracellular sodium and glucose concentrations;\textsuperscript{10,90} however, diffusion of the nanosensors away from the site of injection raised concerns regarding their long-term safety.

Limited studies have been performed on intradermal exposure of nanoparticles\textsuperscript{70} and toxicological effects of nanoparticles is dependent upon multiple parameters (i.e. size, material, and surface coating).\textsuperscript{93-95} In previous nanosensor \textit{in vivo} studies, all of the sensing components were entrapped in a sebacate plasticizer and PVC-based particle.\textsuperscript{10,90} Though both of these components have been used in medical grade products and exhibit low toxicity, plasticizer leaching and known carcinogenic effects of vinyl chloride have caused rising safety concerns associated with plasticized PVC in medical devices.\textsuperscript{12,13} Plasticizer-free ion-selective electrodes\textsuperscript{96,97} and optode sensors\textsuperscript{98} have been developed to minimize the effect of plasticizer leaching on sensor lifetime and adverse biological responses. In this work, we report the development of the first biodegradable optode-based nanosensors. Therefore when applied \textit{in vivo}, the nanosensors will ultimately be degraded and cleared from the body regardless of their \textit{in vivo} fate minimizing long-term toxicity. Sodium sensors were selected for proof of concept for
two reasons. First, clinically, hyponatremia affects 30% of elderly patients in nursing homes\textsuperscript{99} and leads to gait disturbances and increased risk of falls in this population;\textsuperscript{100} however, unlike glucose monitors, no at-home sodium monitoring system exists. Second, the sodium sensing mechanism is well understood and mathematically described in the ion-selective optode field and will provide a robust model system for investigation into new sensor designs.\textsuperscript{24,25,101} Briefly, the mechanism involves three main components: a neutral ionophore, a neutral chromoionophore, and a negatively charged additive. When no sodium is present within the system, the chromoionophore is protonated while the negative additive provides charge neutrality within the sensor. As sodium is introduced, the ionophore extracts sodium into the sensor and the chromoionophore deprotonates resulting in a change of fluorescence signal. Based on the changes in signal intensity, the sodium concentration can be determined and sensor response tailored by changing the ratio of components. The biodegradable nanosensors developed here are composed of polycaprolactone (PCL) and citric acid based plasticizer and retain characteristics traditional to optode sensors.

5.3 Materials and Methods

**Materials.** Sodium ionophore X (NaIX), sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate (NaTFPB), and chromoionophore III (CHIII) were all purchased from Fluka (St. Louis, MO). PCL (M\textsubscript{n}≈14,000) was acquired from Aldrich (St. Louis, MO) and sodium chloride (NaCl), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), and polyoxyethylene-polyoxypropylene block copolymer (Pluronic® F-68) were purchased from Sigma (St. Louis, MO). Acetyltri-n-hexyl citrate (Citroflex A-6) was acquired from Vertellus (Indianapolis, IN). 1,2-disteroyl-sn-glycero-3-
phosphoethanolamine-N-[methoxy(polyethylene glycol)-550] ammonium salt in chloroform (PEG 550) was purchased from Avanti Polar Lipids (Alabaster, AL) and acetone (≥ 99.5%) was acquired from Sigma-Aldrich (St. Louis, MO). Spectra/Por® In Vivo Microdialysis Hollow Fibers (Inner diameter: 200 µm, Outer diameter: 280 µm, Molecular Weight Cut-Off: 13 kilodaltons) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA) and phosphate buffered saline (PBS, pH=7.4) was purchased from Life Technologies (Grand Island, NY). As provided by the manufacturer, the composition of PBS was 1.06 mM potassium phosphate monobasic, 155.17 mM sodium chloride, and 2.97 mM sodium phosphate dibasic.

**Nanosensor Formulation and Fabrication.** Prior to nanosensor fabrication, all sensor components were combined into an optode cocktail: 60.1mg Citroflex A-6, 30mg PCL, 0.1mg (1.11mmol kg⁻¹) NaIX, 0.4mg (4.98mmol kg⁻¹) NaTFPB, and 0.1mg (1.93mmol kg⁻¹) CHIII dissolved in 3mL of acetone. This ratio of sensing components was selected based on their tailored response to sodium at physiological extracellular sodium concentrations (135-145 mM). Additional optode cocktails containing 90.3mg Citroflex A-6 or 90 mg PCL both with the same ratio of sensing components were used as controls in the nanosensor degradation experiments. Sodium nanosensors were fabricated using a solvent displacement method derived from previously developed methods. 3mg of PEG 550 was dried onto the bottom of a 2 dram glass vial and then dissolved in 600 µL deionized water. While stirring the PEG 550 /water mixture, 300 µL of optode cocktail was pipette injected into the mixture and then allowed to stir for 10 minutes. After this time, the sensor solution was centrifuged at 12,600 g for 20 minutes (Micromax RF Refrigerated Microcentrifuge, Thermo Electron Corporation, Milford, MA) and the supernatant was removed. The sensors were then washed two more times with 10mM HEPES (pH=7.4) and centrifuged at 12,600 g for 20 minutes. After the last wash, the
sensors were resuspended in 10mM HEPES for characterization. This fabrication method could be scaled-up to generate more sensors depending upon the desired application without loss of calibrated response (data not shown). For example, ten times the amount of sensors could be made by using 30mg PEG 550, 6 ml deionized water, and 3 ml of optode cocktail.

**Nanosensor Characterization**

**Nanosensor Reversibility.** Reversibility was determined by entrapping the nanosensors in Spectra/Por® In Vivo Microdialysis Hollow Fibers. These fibers have a specified inner diameter of 200 µm and a molecular cut-off of 13 kD thus allowing the diffusion of sodium ions and buffer into the fiber while maintaining the nanosensors. Fibers were pre-filled with 20 mg/ml of Pluronic® F-68 in deionized water to prevent adhesion of nanosensors to the fiber walls and then filled with nanosensors. Fiber ends were sealed with epoxy and then glued onto glass coverslips attached to a microscope perfusion system. Images of the nanosensors while under perfusion were acquired on a Zeiss Confocal Microscope (Thornwood, NY). CHIII has three excitation and emission peaks (ex/em: 488 nm/570 nm, 488 nm/670 nm, 639 nm/680 nm). Sensors were imaged using the 488 nm and 639 nm lasers simultaneously to collect the emission intensities at the 570 nm and 680 nm peaks, respectively. The perfusion chamber was alternately filled with 0 mM and 500 mM of NaCl in 10 mM HEPES (pH=7.4) to measure fluorescence response and recovery of the sodium nanosensors. While under perfusion, images were acquired approximately every 11.6 seconds. Images were exported and analyzed using ImageJ software and Matlab. For all samples, an equal size rectangular region of interest containing only fluorescence was selected and all saturated and zero value pixels were removed prior to analysis. The results were not altered by removing the saturated and zero value pixels. The average fluorescence intensities for the 570 nm and 680 nm emission wavelengths were divided for each
sample to get a ratio of the two wavelengths and error was calculated using the laws of error propagation.

**Nanosensor Response.** The response of the nanosensors to sodium was determined as follows. In a optical bottom 96-well plate, 100 µL of nanosensors were placed in wells along with 100 µL of the following solutions: 0 M, 0.020 M, 0.050 M, 0.1 M, 0.2 M, 0.240 M, 0.270 M, 0.290 M, 0.320 M, 0.380 M, 0.4 M, 0.5 M, 1 M, 2 M of NaCl in 10mM HEPES buffer solution (pH=7.4). As a result of mixing these concentrations of NaCl with the sensors, the total sodium concentration in each of the well is half as stated above. Each concentration was performed in triplicate from three different sets of nanosensors. Fluorescence endpoint measurements were only taken at the excitation and emission wavelengths of 640nm and 680nm, respectively, since emission intensities at the other two wavelengths are not high enough to provide adequate signal to noise. Fluorescence measurements were acquired on a Molecular Devices SpectraMax Gemini EM (Sunnyvale, CA). To determine the response of the sensors, the log of the concentration was plotted against α:\[^{87}\]

\[
\alpha = \frac{I_{[Na^+]}}{I_{[Na^+]}} - \frac{I_{[Min]}}{I_{[Max]}} - \frac{I_{[Min]}}{I_{[Max]}}
\]

where \(I_{[Na^+]}\) is the intensity of the sensors at a specific sodium concentration, \(I_{[Minimum]}\) is the intensity at 1M NaCl and \(I_{[Maximum]}\) is the intensity at 0 M NaCl sodium concentration. The error was determined from the raw fluorescence intensities according to the laws of error propagation. The center of the dynamic range (K_D) was determined as the sodium concentration where \(\alpha=0.5\) and the sensitivity was calculated as the change in sodium concentration for a 1% change in normalized fluorescence signal. In addition, nanosensor response was measured while in the presence of a high background solution of potassium chloride (KCl), a common sodium sensor.
interferent. All NaCl solutions listed above were made in a 30 mM KCl in 10 mM HEPES solution (pH=7.4). The total KCl concentration in each well was 15 mM which is three times greater than extracellular KCl concentrations (3.5-5.0 mM). Each concentration was performed in triplicate for three different sets of nanosensors. Response of the nanosensors was determined similar to described above and a two-tailed student’s t-test was used to determine significant changes in response while in the presence of potassium ions.

Nanosensor Lifetime and Stability. Three parameters were used to determine the lifetime and stability of sodium nanosensors: calibrated response, size, and zeta potential. Each of these parameters was measured on Day 0, 3, 7, 10, and 14. Between measurements, the sensors were stored in an incubator at 37°C to mimic physiological conditions. At each time point, sensors were calibrated using the same conditions, solutions, and methods as described above. The size and zeta potential of the nanosensors was measured using a Brookhaven 90 Plus Particle Size Analyzer (Holtsville, NY). Sensors were diluted in PBS for all size and zeta measurements. The size was taken as the effective diameter that is derived from the measured intensity of the particles.

Scanning Electron Micrographs (SEM). Nanosensors were diluted in deionized water and dried under vacuum on aluminum specimen mounts. We acquired images on a Hitachi S4800 at low accelerating voltages and did not coat the samples to prevent heat damage to the particles.

Nanosensor Degradation. PCL nanoparticles have been shown to be stable in buffer solution for over 140 days, but they are rapidly degraded in the presence of lipases. Nanosensor degradation was tested in the presence of Pseudonamas lipase at 30 U/L, 190 U/L, and 6,000 U/L representing low serum, high serum, and liver levels of lipase respectively. Since our particle is a two component system, degradation of nanosensors
composed of 100% PCL and 100% Citroflex A-6 were also investigated along with our 2:1 Citroflex A-6: PCL nanosensors. Two independent parameters were used to evaluate degradation: fluorescence intensity in 0 mM NaCl and particle count rate. For fluorescence intensity, measurements were acquired on a Molecular Devices SpectraMax Gemini EM (Sunnyvale, CA) at excitation and emission wavelengths of 640 nm and 680 nm, respectively. In a 96-optical bottom well plate, 100 µL of nanosensors were mixed with 100 µL of 0 mM NaCl solution. Count rate was measured on a Brookhaven 90 Plus Particle Size Analyzer. Count rate is proportional to particle concentration and thus provides a relative change in particle concentration over time. Nanosensors in the absence of lipase acted as a control and initial measurements were acquired using a single control experiment (n=3). Further measurements were acquired at 4 hours, 8 hours, 28 hours, and 74 hours for the control and different lipase concentrations. In between measurements, nanosensors both in the presence and absence of lipase were stored at 37°C. Average fluorescence intensities and count rates were normalized and plotted over time to determine degradation of the nanosensors.

5.4 Results and Discussion

Nanosensor Response Characteristics. As a substitute for PVC, PCL was chosen because it is a popular FDA-approved polymer with applications in sutures, scaffolds, and drug delivery devices.110 PCL is a hydrophobic aliphatic polyester that undergoes hydrolytic degradation either through bulk or surface erosion depending upon water penetration into the polymer.110 Furthermore, its rate of degradation is dependent upon molecular weight, pH, and shape.111,112 The plasticizer, Citroflex A-6, was selected as a replacement for DOS because citric acid esters
are biodegradable and are used in medical plastics as a replacement for phthalates. Typically, response characteristics of the sensors such as the dynamic range and selectivity are primarily dictated by the incorporated sensing components and the polymer matrix is considered inert. However, extraction of analytes, leaching, and loading efficiency of the sensors are influenced by the polymer matrix which varies by hydrophobicity and charge depending upon the selection of matrix components. Therefore, response characteristics had to be evaluated for the biodegradable nanosensors even though their sensing mechanism is well-characterized.

The dynamic fluorescence response of the nanosensors was tested by encapsulating the nanosensors in hollow fiber dialysis tubing and attaching the tubing to a perfusion system (Figure 5.1). With increased sodium concentrations, the 570 nm emission peak increases in intensity (Figure 5.1A, Video S-1) and the 680 nm emission decreases in intensity (Figure 5.1B, Video S-2) as CHIII becomes deprotonated. Because of the dual emission response, the fluorescence intensity at each wavelength can be ratioed, increasing sensor sensitivity and minimizing the effects of photobleaching. Figure 5.1C shows the average ratioed fluorescence response of three sets of sodium nanosensors and their rapid fluorescence response and recovery to changes in sodium concentrations. From the response curve, the ratio changes by approximately 0.5 or 70% and the average response time of the sensors, as measured as the time it takes the sensors to reach 95% of their total response to sodium, is 48 seconds. This is the response time of the entire system and takes into account the perfusion time of solutions into the chamber, diffusion of sodium into the dialysis tubing, and response time of the sensors. Since extracellular sodium dynamics are expected to change on a slower timescale than intracellular dynamics, the measured response time is adequate for in vivo extracellular measurements.
Figure 5.1. **Fluorescence reversibility of biodegradable sodium nanosensors.**

Fluorescent confocal images of nanosensors in hollow fiber dialysis tubing at (A) 570 nm emission and (B) 680 nm emission for one set of nanosensors. (C) Reversibility of sodium nanosensors for two cycles. Emission intensities at 570 nm and 680 nm were ratioed at each time point. Error bars represent standard deviations from 3 individual sets of sodium nanosensors. Positive error bars are shown here for clarity, but error has a symmetrical distribution around the mean. Note: A ratio of fluorescence intensities is displayed instead of $\alpha$. 


Figure 5.2 shows the tailored response of the sodium nanosensors with a measured $K_D$ of 141 mM NaCl and sensitivity of 4.6 mM for a 1% change in fluorescence intensity. These values did not significantly shift ($p > 0.01$ as determined from $\alpha$ values) when in the presence of over three times the extracellular potassium levels (15 mM KCl), a common sodium sensor interferent (Figure 5.2). Physiological concentrations of extracellular sodium are between 135 mM and 145 mM. Currently, the center of the dynamic range of our sensors falls within these values, but the sensors are not yet sensitive to accurately monitor sodium concentrations in this range. Future work will focus on improving the sensor sensitivity by tailoring the ratio of components. Additionally, the sensor is inherently susceptible to changes in pH. Therefore, monitoring pH independently or holding the pH constant is necessary for improved accuracy.

For our particular application, measurement in the dermal space, pH is fairly constant, but our results could be easily confounded if particles are endocytosed and exposed to an acidic lysosomal environment. Figure 5.3 shows the lifetime and stability of the biodegradable nanosensors while in buffer solution at 37°C. The calibrated sensor response, size, and zeta potential remain stable over a 14 day time period. The average size of the nanosensors as determined using dynamic light scattering was 260±2.2 nm with an average polydispersity index (PdI) of 0.120±0.02. The presence of larger sensors was visible on SEM (Figure 5.4), but filtering techniques can be used to narrow the size distribution if desired.

**Biodegradation of Sodium Nanosensors.** Once *in vivo*, the expected lifetime and stability of sodium nanosensors will decrease because of physiological and immune responses spurred by their injection. PCL was selected instead of poly(lactic-co-glycolic acid) (PLGA) because its degradation products do not alter the pH of the surrounding system and thus is not expected to alter sensor response. Since our nanosensor particle is a two component system, we
Figure 5.2. Response curves of biodegradable sodium nanosensors with no interferent (■) and with 15 mM KCl background interferent solution (○). There was no significant change in response of the nanosensors to sodium while in the presence of KCl (p > 0.01). Measurements were taken in triplicate for three different sets of nanosensors (n=9). Error bars were calculated using the laws of error propagation.
Figure 5.3. Lifetime and stability of biodegradable sodium nanosensors.  (A) Response curves on Day 0 (---■---), Day 7 (--○--), and Day 14 (···▲···). Measurements were taken in triplicate for three different sets of nanosensors (n=9) and error bars were calculated using the laws of error propagation. (B) Average sizes (■) and zeta potentials (○) measured intermittently over the course of two weeks. Three samples were run from three individual sets of nanosensors. For each sample of nanosensors, 3 size measurements were made and five zeta potential measurements were made. Therefore, n=27 for size measurements and n=45 for zeta potential measurements. Error bars were calculated using the laws of error propagation.
Figure 5.4. SEM micrograph of a biodegradable sodium nanosensor.
investigated the individual degradation response of PCL and Citroflex A-6 independently. Nanosensors composed of only PCL showed both a decrease in fluorescence response and count rate over time (Figure 5.5). On the contrary, sensors composed of only Citroflex A-6 did not show a decrease in fluorescence intensity nor count rate (Figure 5.5). Figure 5.5 also shows the degradation response curves of 2:1 Citroflex A-6: PCL system in the presence of lipase. For all cases, the fluorescence intensity remained constant while the count rate decreased. Based on these results, the sensors had degraded, but the remaining plasticizer sequestered the sensing components resulting in no change in fluorescence intensity over time. Citrates are biodegradable esters\textsuperscript{13} and we expect that Citroflex A-6 also experienced biodegradation by the lipases, but this process could not be monitored using our current techniques. In the first 4 hours, the rate of decrease in count rate was similar at both high serum and liver lipase concentrations and slower at low serum levels. However, the degradation rate is expected to be faster \textit{in vivo} because of optimal enzyme operating conditions and the presence of other degradation mechanisms.\textsuperscript{103}

### 5.5 Conclusion

We have designed biocompatible and biodegradable sodium nanosensors composed of PCL and Citroflex A-6. These sensors have favorable characteristics for \textit{in vivo} applications such as fast and dynamic fluorescence response tailored to sodium levels. The sensors degrade in the presence of lipases with accelerated degradation rates at concentrations that would be found in the liver, a primary bioaccumulation site for nanoparticles. Future work will investigate the toxicity of the degraded nanosensor components including the active sensing components
Figure 5.5. Degradation profiles of sodium nanosensors composed of 100% PCL (■, □), 100% Citroflex A-6 (●, ○), and 2:1 Citroflex A-6 to PCL (▲, Δ). Decreases in both fluorescence intensity (—, solid objects) and count rate (- - -, open objects) were used to assess degradation of sodium nanosensors while in the presence of (A) no lipase, (B) low serum concentrations of lipase (30 U/L), (C) high serum concentrations of lipase (190 U/L), and (D) liver lipase concentration (6,000 U/L). Averages of fluorescence intensity (n=6) and count rate (n=9) are shown with error bars calculated using the laws of error propagation.
that will be released upon degradation. Though this system has been developed for sodium, the new sensor platform can be extended by exchanging the sensing components to monitor other analytes such as potassium and chloride, small molecules such as glucose, or even larger molecules such as enzymes.

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### 5.6 Acknowledgements

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Chapter 6: Optimization of Glucose Sensor Components

6.1 Chapter Overview

For clinical applications, the sensitivity and long term stability of glucose-sensitive nanosensors must be improved. In this chapter, more advanced boronic acid sensing moieties and alizarin-based fluorescent dyes were incorporated into the sensors. Boronic acids containing electron withdrawing functional groups responded to glucose greater than our current formulation; however, future work will still involve optimizing sensor response and functionalizing fluorescent dyes for improved lifetime.

6.2 Introduction

Independent of in vivo obstacles, fluorescent glucose-sensitive nanosensors have two key limitations to clinical applications: leaching of sensing components and a limited dynamic range that is insensitive to hypoglycemic events. For example, alizarin is much more hydrophilic than the ionophores and chromoionophores from standard optodes and it leaches quickly from the hydrophobic polymer. As shown in Chapter 3, leaching of sensor components limited in vitro nanosensor lifetime to less than 8 hours. Miniaturization of glucose sensors into nanosensors accelerated leaching of sensor components because of the increased surface area to volume ratio of nanoparticles. Thus, direct conjugation of the sensor components to the nanosensors may prove to alleviate these concerns.

Changing the boronic acid sensing moiety can be used to adjust the sensor dynamic range. The response of the sensors to glucose is governed by the formation of the boronic acid to a boronate ester. Boronic acids have pK$_a$ values that limit boronate ester formation at neutral
pH. The addition of electron withdrawing groups has been shown to decrease the pKₐ value of boronic acids and concurrently increase glucose binding capacity at neutral pHs. For example, Asher and coworkers incorporated fluoro-containing boronic acids into photonic crystal glucose-sensing material because fluorinated boronic acids have pKₐ values at physiological pH levels. Incorporation of boronic acids with electron withdrawing groups will improve sensor dynamic range and sensitivity within physiological levels. However, addition of these groups typically comes with increased hydrophilicity, a trait that leads to leaching from and incompatibility with the hydrophobic sensor platform.

Derivitization of alizarin and boronic acids with hydrophobic chemistries or conjugation to the polymeric sensor platform will mitigate component leaching while incorporating functional groups that enhance glucose response at physiological conditions. In this chapter, alizarin derivatives with carboxyl and amine functional groups were tested with sensor components because of alizarin’s current use in the sensor design and carboxyl and amine groups allow for attachment to the polymeric platform or hydrophobic chains. Boronic acids with electron withdrawing groups and functional chemistries were tested as well because of their known binding with glucose at physiological pH.

6.3 Materials and Methods

**Materials.** Poly(vinylchloride) carboxylated (>97% GC) (PVC-COOH), bis-(2-ethylhexyl)sebacate (DOS), polycaprolactone (Mₙ 70,000-90,000)(PCL), tridodecylmethylammonium chloride (TDMAC), alizarin, alizarine claret (mordant dye), alizarin-3-methyliminodiacetic acid, 3-fluoro-4-methoxycarbonylphenylboronic acid, D-(+)-glucose, tetrahydrofuran (≥ 99.9%) (THF), dicyclohexylcarbodiimide solution (60 weight
percent in xylenes) (DCC), and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich (St Louis, MO, USA). Octylboronic acid (>97%) and Citroflex A-6 were acquired from Synthonix (Wake Forest, NC, USA) and Vertellus (Indianapolis, IN, USA), respectively. Phosphate Buffered Saline (PBS) (1x, pH = 7.4) was purchased as a solution from Invitrogen (Carlsbad, CA, USA).

**Polymer Functionalization.** Figure 6.1 shows the reaction mechanism for attaching alizarin-3-methyliminodiacetic acid to polyvinyl chloride with amine groups. In 1 ml of THF, 6 mg of alizarin-3-methyliminodiacetic acid and 3 mg of NHS were dissolved. Under stirring, 10 μL of DCC in xylene was added to the mixture and then reacted for 6 hours. After this time, 50 mg of polyvinyl chloride amine in 2 ml THF was added to the alizarin-3-methyliminodiacetic/NHS/DCC mixture and stirred overnight. The reaction mixture was then centrifuged for 20 minutes at 12,600g to separate out the dicyclohexyl urea precipitate. THF was removed from the polymer solution and the dried polymer was washed three times with methanol to remove unreacted starting materials.

**Fluorescent Dye Response to Boronic Acids.** All samples were prepared in quartz cuvettes with a total volume of 2.5 ml THF. For each dye, the components and details for fluorescence measurements are in Table 6.1. For each titration, boronic acids were added at increasing concentrations, the solution was mixed, and then fluorescence measurements were acquired. Octylboronic acid was titrated with alizarin and alizarine claret and 4-amino-3-fluoro-phenylboronic acid bound to PVC-COOH was titrated in with alizarin-3-methyliminodiacetic acid. Controls for dilution effects of each dye involved titrating in an equal amount of volume of the control solution.
Figure 6.1. Synthesis of alizarin-3-methyliminodiacetic acid (1) conjugated to polyvinyl chloride containing amine functional groups (2).
Table 6.1. Experimental set-up for detecting fluorescence changes of dyes in the presence of boronic acids.

<table>
<thead>
<tr>
<th>Cuvette Solution</th>
<th>Alizarin</th>
<th>Alizarine Claret</th>
<th>Alizarin-3-methyliminodiacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.10 mg alizarin</td>
<td>0.05 mg alizarin claret</td>
<td>1.50 mg alizarin-3-methyliminodiacetic acid-PVC</td>
</tr>
<tr>
<td></td>
<td>0.22 mg TDMAC</td>
<td>0.11 mg TDMAC</td>
<td>(0.125 mg alizarin-3-methyliminodiacetic acid)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.27 mg TDMAC</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Ratios of Dye to Boronic Acid</th>
<th>Alizarin</th>
<th>Alizarine Claret</th>
<th>Alizarin-3-methyliminodiacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
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<td>1:0</td>
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<tr>
<td>1:1</td>
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<table>
<thead>
<tr>
<th>Control Solution</th>
<th>THF</th>
<th>THF</th>
<th>PVC-COOH in THF</th>
</tr>
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</table>
**Polymer Composition of the Optode.** Formulation 1 was made from the following components: 30 mg high molecular weight PVC-COOH, 60 μl DOS, 3.0 mg octylboronic acid, 4.0 mg TDMAC, and 1.0 mg alizarin. These materials were charged into a glass vial and then dissolved in 500 μl THF. Formulation 2 was made from the following components: 30 mg PCL, 60 μl Citroflex A-6, 16.5 mg 3-fluoro-4-methoxycarbonylphenylboronic acid, 2.0 mg TDMAC, and 1.0 mg alizarin. These materials were charged into a glass vial and then dissolved in 500 μl THF.

**Reversibility of the Macrosensor.** Reversibility was determined similar to Chapters 2 and 3. Data was acquired in a Spectramax Gemini EM microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission set to 460 and 570 nm, respectively. Optode (2 μl) of each formulation was pipetted onto the bottom of a 96-well optical bottom plate each containing a glass cover slip. The optodes were then allowed to dry at least 15 minutes forming macrosensors. Each optode was hydrated in 200 μL PBS (pH=7.4) overnight. After the optodes were hydrated, the PBS solution was removed from all wells and 200 μl of 0.1 M glucose in PBS was pipetted into half of the wells. The remaining wells acted as controls and contained fresh glucose-free PBS. In order to track the changes in the fluorescence response, measurements were acquired at a sampling rate of 5 minutes for 60 minutes. Then, all solutions (glucose and control) were removed and 200 μl of fresh PBS was added to all wells. Again, measurements were obtained every 5 minutes for 60 minutes. Response was determined by the percent change difference between the average intensity of the glucose and control. The fluorescence intensity of each sensor was normalized to time zero and then the average was taken for each experimental group. The average of the experimental group was subtracted from the control group and multiplied by 100 to obtain a percent change. The error was calculated using error propagation.
**Calibration of the Macrosensor.** Data was acquired in a Spectramax Gemini EM microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). Macrosensors were fabricated using the method above. Each macrosensor/optode was hydrated in 200 µL PBS (pH=7.4) overnight. At the end of this period, the PBS solution was removed and another 200 µL of fresh PBS was added to the wells. In order to track the changes in the fluorescence response, measurements were acquired at a sampling rate of 5 minutes for 60 minutes. The PBS solution was then replaced with 200 µl of 1 mM glucose in PBS (pH = 7.4) for the experimental wells and fresh PBS for the control wells. The optodes were allowed to equilibrate again for 60 minutes. The process was repeated for 5 mM, 10 mM, 30 mM, 50 mM, and 100 mM in PBS. The fluorescence of each sensor was normalized to the first time point of 0 mM glucose. The normalized response of all sensors within each group at the 60 minute time point was averaged. The average of the experimental group was then subtracted from the control group. The difference was multiplied by 100 to express the difference in response as a percent. The percent difference was plotted against the log of the glucose concentration.

**6.4 Results and Discussion**

**Fluorescent Dye Response to Boronic Acids.** Several key criteria were used to assess new fluorescent dyes for incorporation into glucose-sensitive nanosensors. First, the dye must contain a 1,2-diol which experiences a change in fluorescence intensity upon binding to the boronic acid sensing moiety. Figure 6.2A shows an increase in fluorescence intensity of alizarin, the currently used dye, in the presence of increasing boronic acid concentration. This binding must be reversible for dynamic glucose sensing. Second, the dye must be hydrophobic to minimize leaching. Third, the dye must have red shifted fluorescence emission to be visualized.
Figure 6.2. Emission spectra of fluorescent indicators in the presence of increasing concentrations of boronic acid. (A) Alizarin emission at ratios of 1:0 (■, black), 1:1 (●, red), 1:3 (▲, blue), and 1:6 (▼, teal) alizarin:octylboronic acid. (B) Alizarine claret emission at ratios of 1:0 (■, black), 1:1 (●, red), 1:3 (▲, blue), and 1:6 (▼, teal) alizarin:octylboronic acid. (C) Alizarin-3-methyliminodiacetic acid-PVC emission at ratios of 1:0 (■, black), 1:9 (●, red), 1:17 (▲, blue) alizarin-3-methyliminodiacetic acid:4-amino-3-fluoro-phenylboronic acid bound to PVC-COOH. All ratios were calculated based on number of molecules.
through the skin. To meet these requirements, two diol containing dyes were tested: alizarine claret and alizarin-3-methyliminodiacetic acid. Alizarine claret has red shifted fluorescence emission and amine groups for potential conjugation to the polymeric sensor platform. Direct conjugation of the dye would prevent dye leaching over longer periods of time. However, Figure 6.2B shows that in the presence of increasing boronic acid concentration, alizarine claret does not undergo a resulting change in fluorescence intensity. Additionally, several other boronic acids were tested, but none were able to cause a change in alizarine claret fluorescence indicating that either boronic acids cannot bind to the dye or no change occurs upon binding (data not shown). Similar to the standard alizarin (Figure 6.2A), alizarin-3-methyliminodiacetic acid demonstrated an increase in fluorescence intensity with increasing boronic acid concentration (Figure 6.2C). Alizarin-3-methyliminodiacetic acid also contains two free carboxylic acid groups that were further used for functionalization and conjugated to polyvinyl chloride amine. However, when incorporated into sensors, the sensors did not respond to glucose. The absence of response may be caused by insufficient concentrations of alizarin-3-methyliminodiacetic acid conjugated to the polyvinyl chloride or inhibited dye mobility when the polymer is not in solution. A summary of each dye is presented in Table 6.2. Since neither alizarin-3-methyliminodiacetic acid or alizarine claret was an improvement over alizarin, alizarin was used in all future studies.

**Boronic Acid Selection.** The presence of electron withdrawing groups on boronic acids has been cited as a way to improve boronic acid binding to diols at physiological pH levels. Octylboronic acid is currently the sensing moiety incorporated in the glucose-sensitive nanosensors. Though octylboronic acid can sense glucose at pH 7.4, its response to glucose dramatically increases at pH 12 (data not shown). Octylboronic acid contains no electron
Table 6.2. Summary of fluorescent dye characteristics tested for glucose-sensitive sensors.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Alizarin</th>
<th>Alizarine Claret</th>
<th>Alizarin-3-methyliminodiacetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Structure</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td><img src="image3" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Excitation/Emission Wavelengths (nm)</td>
<td>460/570</td>
<td>530/610</td>
<td>460/570</td>
</tr>
<tr>
<td>Pros</td>
<td>Changes fluorescence in presence of boronic acids</td>
<td>Red-shifted emission spectrum for <em>in vivo</em> visualization</td>
<td>Changes fluorescence in presence of boronic acids</td>
</tr>
<tr>
<td></td>
<td>Presence of amine group for functionalization</td>
<td>Presence of carboxyl groups for functionalization</td>
<td>High fluorescence intensity</td>
</tr>
<tr>
<td>Cons</td>
<td>Emission within the range of autofluorescence</td>
<td>Does not change fluorescence in presence of boronic acids</td>
<td>Emission within the range of autofluorescence</td>
</tr>
<tr>
<td></td>
<td>No functional groups available for further chemical modification</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
withdrawing groups, but was selected because its hydrophobicity is compatible with our sensor platform. However, at this point in sensor design, sensor dynamic range and sensitivity needs improvement to meet clinical application criteria. Therefore, boronic acids with different electron withdrawing groups (i.e. nitro, fluoro, carboxyl) and additional functional groups for future chemical modifications were tested (data not shown). Of the compounds tested, phenyl boronic acids with fluorinated groups attached to the phenyl ring showed the greatest fluorescent response to glucose at pH 7.4. Figure 6.3 shows the response to glucose with sensors containing 3-fluoro-4-methoxycarbonylphenylboronic acid compared to our traditional glucose sensors. Figure 6.3A shows fluorescence response of sensors to 100 mM glucose and then fluorescence reversibility when glucose was removed. Figure 6.3B shows the fluorescence response of glucose sensors to increasing glucose levels up to 100 mM glucose. The new sensors yielded a 5% response to 100 mM glucose with a calibrated response to glucose. In contrast, our traditional glucose sensors with octylboronic acid showed approximately a 0.5% change in fluorescence intensity to 100 mM glucose. The new sensor formulation with 3-fluoro-4-methoxycarbonylphenylboronic acid can be improved by tailoring the ratio of components for greater response in the physiological range. Table 6.3 compares the two sensor formulations based on important sensor characteristics. In addition to a greater response, the updated formulation includes the newly developed biodegradable sensor platform detailed in Chapter 6. A drawback of the updated sensor formulation is that 3-fluoro-4-methoxycarbonylphenylboronic acid does not have a fully reversible fluorescence response to glucose. Full recovery of the signal may be prevented by the boronic acid binding tightly to glucose or leaching of boronic acid from the sensor. Future work involves modification of the carboxyl functional group such
Figure 6.3. Response of fluorescent sensors to glucose. (A) Response and reversibility of sensor response to 100 mM glucose of Formulation 1 (■, n_{control}=8, n_{experimental}=8) and Formulation 2 (○, n_{control}=8, n_{experimental}=8). and (B) calibration curve of Formulation 1 (■, n_{control}=6, n_{experimental}=5) and Formulation 2 (○, n_{control}=8, n_{experimental}=8). Error bars are calculated using the rules of error propagation.
Table 6.3. Summary of progress in sensor formulation based on several key design parameters.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Previous Formulation</th>
<th>Updated Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensing Moiety</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>![Chemical Structure]</td>
<td>![Chemical Structure]</td>
</tr>
<tr>
<td>Sensing Platform</td>
<td>PVC-COOH, DOS</td>
<td>PCL, Citoflex A-6</td>
</tr>
<tr>
<td>Response to Glucose</td>
<td>≤ 1% response at pH 7.4 and 0.1M glucose</td>
<td>5% response at pH 7.4 and 0.1M glucose</td>
</tr>
<tr>
<td>Reversibility</td>
<td>Reversible</td>
<td>Not fully reversible</td>
</tr>
<tr>
<td>Biocompatibility</td>
<td>Concerns with plasticized PVC medical products</td>
<td>FDA-approved and biodegradable components</td>
</tr>
</tbody>
</table>
as extension of the methyl group to longer alkane chains to improve boronic acid hydrophobicity.

6.5 Conclusions

Glucose-sensitive nanosensors demonstrated a poor dynamic range of detection and leaching of sensor components. Therefore, alternatives to the sensing moiety and fluorescent dye were tested. The alizarin derivative, alizarin-3-methyliminodiacetic acid, demonstrated strong changes in fluorescence in the presence of boronic acids and contained functional groups for conjugation to the polymeric platform. However, when incorporated into sensors, the conjugated dye did not yield a response to glucose. In contrast, selection of fluorinated boronic acids improved the response of the sensors over the previous formulation, but chemical modification of these boronic acids is still necessary to prevent leaching from the nanosensors.

6.6 Acknowledgements

I would like to thank Yi Luo for his help in boronic acid and fluorescent dye testing and functionalizing these components to polymers. Funding for this project was provided by Northeastern University’s internal Tier 1 grant.
Chapter 7: Electrospun Nanofibrous Sensors for Analyte Detection

7.1 Chapter Overview

In initial in vivo studies (Chapters 3 and 4),\textsuperscript{117,118} nanosensor migration away from the site of injection limited their monitoring time. In this chapter, fibrous scaffolds composed of glucose sensor components were fabricated with electrospinning in order to generate scaffolds which are large enough to eliminate migration, but small enough to retain reasonable response time. Scaffolds with fibers less than 5 μm in diameter were produced and yielded an optical response to glucose. Future work will involve testing the scaffold lifetime in vivo.

7.2 Introduction

Preliminary in vivo experiments show the feasibility of fluorescent glucose-sensitive nanosensors to track changes in glucose levels,\textsuperscript{119} but short in vivo lifetimes due to particle migration away from and cellular uptake at the injection site have stagnated the progress of these nanosensors as an in vivo monitoring tool. Various approaches have been used to overcome these issues such as nanosensor immobilization into gels (Chapter 4)\textsuperscript{118} and altering sensor geometry.\textsuperscript{71} Gel immobilization extended sensor lifetime at the injection site over the course of hours, but did not provide a long-term solution to sensor migration.\textsuperscript{118} As an alternative to the gels, Dubach et al. showed that changing sensor geometry from spherical to cylindrical (microworms) prevented intensity loss at the injection site over the course of one hour.\textsuperscript{120} However, their fabrication was tedious and did not result in a high sensor yield for sufficient in vivo signal or larger in vivo studies.
Electrospinning is a commonly used, high rate, and scalable manufacturing technique for making nanofibrous scaffolds. This technique increases sensor size relative to nanosensors while retaining the advantageous features of nanoparticle and microworm geometry such as fast response times. Electrospinning uses high voltages to charge a polymer solution that produces fibers accelerating towards ground. This process produces fibers that range in diameter from several nanometers up to 5µm. The fiber diameter can be controlled by adjusting the polymer concentration in the solution, solvent selection, solution conductivity, and solution flow rate. Additionally, fiber alignment and scaffold porosity can also be changed for example by using a rotating drum collector and ultrasonication post modification, respectively. Electrospun scaffolds have high surface area to volume ratios and porosities between 80 and 90% that will allow for sufficient diffusion of extracellular fluid and fast response times of the fibers. Other groups have utilized electrospinning to fabricate sensors for the detection of silver, mercury, nitroaromatics, and glucose. The sensors previously developed, however, were only tested in vitro. This research sets the groundwork for eventual in vivo application.

7.3 Materials and Methods

Materials. Polycaprolactone (Mₙ 70,000-90,000)(PCL), tridodecylmethylammonium chloride (TDMAC), alizarin, 3-fluoro-4-methoxycarbonylphenylboronic acid (3F4MOCPBA), D-(-)-glucose, tetrahydrofuran (≥ 99.9%) (THF) were purchased from Sigma Aldrich (St Louis, MO, USA). Citroflex A-6 was acquired from Vertellus (Indianapolis, IN, USA). Phosphate Buffered Saline (PBS) (1x, pH = 7.4) was purchased as a solution from Invitrogen (Carlsbad, CA, USA).
**Optode Cocktail.** For production of electrospun scaffolds, the general optode cocktail was made with a solution of 12% (weight/volume) of PCL and Citroflex A-6. Of this weight percentage, 10% is Citroflex A-6. Specifically, the optode formulation was: 216 mg PCL, 24 µL Citroflex A-6, 16.5 mg 3F4MOCPBA, 2 mg TDMAC, and 1 mg alizarin in 2 ml THF.

**Fabrication of Fibrous Scaffolds.** Electrospinning was performed on a Nanospinner NE 200 instrument equipped with a syringe pump (Inovenso). The optode solution was spun at a distance of 10 cm from the collector with a rate of 3 ml/hr and at an acceleration voltage of 15 kV. The fibers were spun onto either aluminum foil or silanized glass discs attached to aluminum foil for imaging and testing scaffold response.

**Scaffold Response to Glucose.** To determine scaffold response to glucose, scaffolds spun onto glass discs were removed from the aluminum foil using a 6 mm biopsy punch (Miltex, Inc.) and placed in a 96-well optical bottom well plate. 200 µL of PBS was added to each well and the sensors were hydrated in PBS overnight. All fluorescence measurements were acquired at 460 nm and 570 nm for excitation and emission wavelengths, respectively using a SpectraMax Gemini EM plate reader. After hydration, 200 µL of PBS (pH 7.4) as a control and 100 mM glucose in PBS (pH 7.4) were used to replace the hydration solution. The fluorescent response of the scaffolds was monitored for 2 hours at 5 minute intervals. To determine sensor reversibility, the solutions were removed and PBS was placed into all wells (control wells and experimental wells) after 2 hours. The fluorescence response was then monitored for 1 hour at 5 minute intervals. Fluorescence measurements were normalized to the first time point, averaged for each experimental group, and then plotted over time.

**Fluorescence Imaging.** Images of scaffolds were acquired on a Zeiss Confocal Microscope (Thornwood, NY).
SEM Acquisition. Images were acquired on a Hitachi S4800 with a 5kV accelerating voltage. Samples were not sputter coated.

7.4 Results and Discussion

Electrospinning is a high rate manufacturing technique that produces scaffolds composed of nano and microfibers. In this work, the benefit of the fibrous structure is two-fold: 1) scaffold porosity and microstructure does not limit sensor response time and 2) increased surface of the scaffold will prevent sensor migration and cellular uptake. Images of the scaffold taken using confocal microscopy and SEM are shown in Figure 7.1A and 7.1B, respectively. Both images show the scaffold structure and that a majority of fibers were below 5 μm. When the plasticizer content was 10%, common fiber defects such as beading and wetting were not present throughout the scaffold; however, as the plasticizer was increased to 30% continuous fibers were not produced (data not shown).

Figure 7.2 shows the response of glucose-sensitive scaffolds composed with sensor components optimized in Chapter 6. The scaffolds experienced a less than 10% change in fluorescence compared to the control. Reversibility of sensor fluorescence was not achieved due to the selection of boronic acid as mentioned in Chapter 6. Though the scaffolds responded to glucose, their response time was over an hour. Fiber size of less than 5 μm should yield faster response times. The slow response time may be due to mass transport effects of glucose throughout the scaffold. Transport of glucose can be improved by altering the experimental setup from a static well-plate environment to a continuous flow chamber testing apparatus. Response times of less than 5 minutes are necessary to match commercially-available glucose
Figure 7.1. Electrospun glucose-sensitive scaffolds. (A) Confocal and (B) SEM images show that fibers are less than 5 µm.
Figure 7.2. Response of glucose-sensitive scaffolds to glucose. Scaffolds were exposed to either PBS only (■) or 100 mM glucose in PBS (○). After 2 hours, solutions were removed and all scaffolds were exposed to PBS. Error bars represent standard deviation of normalized values and only negative error bars are shown for clarity ($n_{\text{control}} = 6$ and $n_{\text{glucose}} = 8$).
monitors. The low plasticizer content of the scaffold may have contributed to the slow response time and increasing plasticizer content can improve response. However, PCL-based electrospun scaffolds are rarely produced with plasticizers and even the effect of non-traditional plasticizers such as water is known to affect fiber mechanical stability.\textsuperscript{128} Therefore a trade-off exists between optimal sensor response and fiber integrity. If higher plasticizer contents are required, then switching to a higher molecular weight PCL or changing to a different polymer may be necessary. For example, electrospun nanofibers have been fabricated with ethyl cellulose with up to 40\% plasticizer demonstrating that high percentages of plasticizer can be used.\textsuperscript{124} The slow response time of the fibers may be improved by fabricating thinner scaffolds that allow for faster diffusion of glucose throughout the scaffold. Additionally, the current scaffold design contains only 10\% plasticizer content in contrast to 66\% plasticizer in traditional optode-based sensors. The highly plasticized matrix aids the diffusion of sensor components and analytes within the sensor.

### 7.5 Conclusions

In this chapter, electrospinning fabricated glucose-sensitive scaffolds less than 5 \( \mu \text{m} \) in diameter. The scaffolds responded to glucose, but improvements to sensor design are still required to decrease scaffold response time and scaffold reversibility. In addition to improving scaffold response, future work will also include \textit{in vivo} testing to determine if the new scaffold design lengthens sensor lifetime.

### 7.6 Acknowledgements
I would like to thank Dr. Chris Skipwith for acquiring SEM images of the scaffolds. Funding for this project was provided by Northeastern University’s internal Tier 1 grant.
Chapter 8: Conclusions and Future Directions

The global epidemic of diabetes spurred development of fluorescent sensors to monitor glucose levels minimally invasively. The advantages of fluorescent glucose-sensitive nanosensors designed in this dissertation include fast response times, easy in vivo implantation, non-invasive and continuous measurements, and biodegradable and nonbiological components. The sensor development process began with basic design principles such as glucose extraction into a hydrophobic sensor platform and sensor response at physiologically relevant concentrations (Chapters 2 and 3). After these basic requirements were achieved, the sensor design evolved to meet stringent requirements for monitoring glucose levels in vivo: sensor sensitivity (Chapter 6), lifetime (Chapter 6), immobilization (Chapters 4 and 7) and biocompatibility (Chapter 5). To this end, the sensor was transformed into a biodegradable platform composed of the FDA approved polymer, PCL (Chapter 5). The sensors degrade into biologically safe components in the presence of degradative enzymes (Chapter 5). For minimizing sensor diffusion in vivo, sensors were encapsulated in hydrogels (Chapter 4) and new sensor geometries (Chapter 7) were explored. Gel encapsulation offered only minimal extension to in vivo lifetime and thus, the sensor platform was transformed from nanoparticle to nanofibrous scaffold (Chapter 7). Scaffold-based sensors were designed that responded to glucose (Chapter 7) and further improvements to the sensing mechanism such as sensor sensitivity were achieved (Chapter 6).

This dissertation highlights the progress and continual design of glucose-sensitive nanosensors, but several obstacles still remain before they are applied to a clinical setting. Currently, the sensors do not respond to changes in glucose levels in the hypoglycemic range.
Inclusion of electron withdrawing groups, such as fluoride, to the boronic acid sensing moiety improved sensor response at physiological pH (Chapter 6), but these sensors were not fully reversible. Hydrophilicity of the boronic acids may cause lack of reversibility and chemical modification of the methyl group on 3-fluoro-4-methoxycarbonylphenylboronic acid into longer alkane chains is being explored to improve lipophilicity while retaining glucose response. Future work will also involve synthesis or chemical modification of diol-containing fluorescent dyes that fluoresce in the near infrared red (NIR). Emission in the NIR is important for *in vivo* monitoring in order to visualize the sensors above the autofluorescence of the skin. Lastly, inclusion of a reference will occur after selection of a new diol-based fluorescent dye. Previous work on including a reference dye was unsuccessful because the broad excitation and emission spectrum of alizarin interfered with the fluorescence of several potential reference dyes (data not included). Ratiometric readings with a reference dye and fluorescent changes of the diol-based indicator will generate sensors capable of quantitative measurements.

In addition to optimizing sensor components, extensive characterization of the sensors beyond what is presented here is necessary. An important criterion for the sensors will be their performance in the Clarke Error Grid Analysis. The Clarke Error Grid is the gold standard for measuring the accuracy of glucose meters. A response within 20% of the reference method is within the clinically acceptable region. Generation of at least an *in vitro* Clarke Error Grid with clinical samples will help to determine if further sensor development is necessary before extensive *in vivo* characterization. Once sensor development has advanced *in vitro*, several key *in vivo* studies should be performed that involve sensor response, sensor lifetime, and sensor biodistribution. Initial *in vivo* studies of the sensors were limited to one hour because of sensor migration away from the site of injection. This limitation prevented sensor tracking of multiple
cycles of hypo and hyperglycemic events in vivo. With nanofibrous scaffolds, sensing lifetime should be extended and thus capture these events. The ultimate goal is for the scaffolds to extend in vivo sensing to at least seven days. Lastly, from a regulatory perspective, the sensors are biodegradable and conducting biodistribution studies will determine if sensor components are accumulating in various organs or being excreted.

The fluorescent glucose sensors presented have applications in both clinical and research settings. As presented in this thesis, the current state of the art for these sensors includes fabrication with biocompatible polymers, a suite of potential dyes, attachment chemistries, and multiple structures from nanoscale to macroscale. The sensors have a controllable response range, lifetime, and response time. Additionally, gel encapsulation and alternative sensor geometries are now available for controlling in vivo sensor migration. This thesis demonstrated the progress in design of glucose-sensitive nanosensors with notable achievements in the optode sensor field that include the first demonstration of sensors for small molecules using only non-biological components and the demonstration of the first biodegradable sensors. With this groundwork complete, future work will optimize sensor components and move preclinical testing of the sensors forward.
Chapter 9: References


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