Implantable Biosensors for Physiologic Status Monitoring During Hemorrhage

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IMPLANTABLE BIOSENSORS FOR PHYSIOLOGIC STATUS MONITORING DURING HEMORRHAGE

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Christian Nikolai Kotanen
August 2013

Accepted by:
Dr. Anthony Guiseppi-Elie, Committee Chair
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ABSTRACT

Trauma diagnostics and management are major aims of research for implantable amperometric enzyme biosensor technology [1-3]. Biosensors are capable of monitoring metabolic variables in a minimally invasive manner [4] and have great potential to augment current wireless vital sign monitoring technologies [5] in order to make a more robust physiologic status monitoring platform [3]. The dual responsive Electrochemical Cell-on-a-Chip Microdisc Electrode Array (ECC MDEA 5037) is a recently developed electrochemical transducer for use in a wireless, implantable biosensor system for the continuous measurement of interstitial glucose and lactate. Hyperglycemia arising from insulin resistance and hyperlactatemia arising from anaerobic metabolism both occur following trauma and hemorrhage. The extent of trauma can be correlated to the magnitude of the hyperglycemic response [6]. Rate of production and final concentration of lactate in both interstitial and blood compartments have also been observed to correlate to various stages and endpoints of trauma [3, 7-9]. Applying the integrated metabolic response of these analytes to direct lifesaving interventions may decrease mortality and other complications of hemorrhage and shock.

The Pinnacle dual potentiostat (A) and MDEA 5037 biotransducer (B) form a system (C) intended to demonstrate the value of advanced point-of-care diagnostic technology. When configured as an ASIC, this minimally invasive implantable device may be used by first responders to initiate early and continuous monitoring of patient physiologic status. Development of the bioactive membrane and both in vitro and in vivo
characterization of the MDEA 5037 have been performed. Using the mediator ferrocene monocarboxylic acid (FcCOOH) as a redox probe, the steady state dose response of the MDEA 5037 was 1.9 times greater than the theoretical response as determined by the Cottrell relationship for multi-disc array electrodes. Electrochemical impedance spectroscopic analysis of the MDEA 5037 fits a standard Randle’s equivalent circuit model with error <9% when probed with FcCOOH. The oxidoreductase enzymes glucose oxidase (GOx) and lactate oxidase (LOx) have been immobilized onto MDEA 5037 transducer working electrodes via electro-co-polymerization of pyrrole (Py) and pyrrole butyric acid (PyBA) (7:1 feed) (p(Py-co-PyBA copolymer). Biotransducers were shown to have a sensitivity of 4.75 (±0.93) µA/mM/cm², a $K_{M_{app}}$ of 7.20 (±2.59) mM, an $I_{\text{max}}$ of 76.75 (25.50) µA/cm², a response time of 16.5 (±5) s, and a detection limit of 0.05 (±0.03) mM. The foregoing use of the p(Py-co-PyBA) co-polymer yielded a fivefold increase in sensitivity (p < 0.001) and a threefold increase in maximum current (p < 0.01) compared to biotransducers fabricated with polypyrrole (PPy) only. A Sprague-Dawley hemorrhage model was instrumented with indwelling biotransducers and externalized wireless potentiostat. Resection after 4 h and subsequent in vitro testing showed a decreased sensitivity from 0.68 (±0.40) to 0.22 (±0.17) µA/mM/cm² with an average change of 56 (±48)% and a change in the in limit of detection from 0.05 (±0.03) to 0.27 (±0.27) mM with an average change of 298 (±298)%. Response time of resected biotransducers was 244 (±193) s, 6 times greater than pre-implanted biotransducers with a response time of 41 (±18). This is likely due to biofouling and/or protease related enzyme denaturation. For long duration implantable devices [10] an electroconductive
hydrogel [11] has been developed to confer its antifouling properties [11, 12]. In addition to mitigating inflammation and fibrous encapsulation, these hydrogels maintain the stability of the enzymes far more effectively when compared to system fabricated with just polypyrrole and show increasing sensitivity during storage for up to 21 days [1].
ACKNOWLEDGMENTS

I would like to thank my advisor, Prof. Guiseppi-Elie, for introducing me to the field of biosensors and bioelectronics. With his guidance and with support from the rest of the lab at the Center for Bioelectronics, Biosensors and Biochips (C3B) I have been able to broaden my knowledge of bioengineering with electrochemistry, biologically responsive smart materials, bioinstrumentation, diagnostic systems and trauma physiology. I would also like to thank Prof. Guiseppi-Elie for his support in the classroom that gave me the necessary foundation for performing research, and giving me the opportunity to co-lecture in a classroom setting in one of his classes. I would like to thank my lab mates Olukayode Karunwi, Nolan Wilson, Subrha Nag, Lorcan Ingham, Ruth Salas, and Fouzan Alam for their input and support. I wish to thank the members of my thesis committee Prof. Robert Latour, Prof. Naren Vyavahare, Prof. Bruce Gao, Prof. Edward Jauch, and Dr. John Parrish for all of their valuable time, feedback and guidance. Finally, I thank my family for all of their love, support and words of encouragement.

Christian N. Kotanen

Monday, July 22\textsuperscript{nd}, 2013.
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CHAPTER ONE: MONITORING SYSTEMS AND QUANTITATIVE MEASUREMENT OF BIOMOLECULES FOR THE MANAGEMENT OF TRAUMA

1.1 Introduction

Hemorrhage is a primary complication of trauma as seen on battlefields, in emergency departments, operating rooms, and intensive care units [13, 14]. Available mortality data shows that, in 2006, unintentional injuries were the leading cause of death in individuals of 1-44 years of age and the 5th over-all leading cause of death for all age groups in the United States [15]. Also, in 2006, for every trauma induced death, there were eleven times as many hospitalizations and 179 times as many emergency department visits [15]. Trauma diagnosis describes the nature of an injury as well as the body region affected by said injury. Injury severity score (ISS) based on both mechanisms and locations of injury is typically used to gauge the threat to life that trauma exhibits [16]. An ISS of ≥16 is considered severe trauma [16].

Discussion of hemorrhage is often difficult because the events of trauma are not fully understood or immediately observable. Overlap of cardiovascular, metabolic, neural, and endocrinal events blur the lines of trauma and hemorrhage related phenomena. Factors of age, fitness, lifestyle, diet, type of trauma, total blood volume (TBV) loss, rate of blood loss, and pre-existing physiologic condition have a cumulative influence on the metabolic and physiologic responses for a trauma patient [17-22]. Severe trauma induced hemorrhage, or blood loss >30-40% total blood volume, is capable of sending an individual into a state of hemorrhagic shock [23]. Shock is defined
as a physiological state of hypoperfusion in which oxygen is inadequately used or delivered at the cellular level [24, 25].

Trauma management involves diagnostics and prognostics that begin in the pre-hospital setting at the site of patient injury and continues throughout the time course of patient care. The goals of trauma management are to predict, prevent, and treat the state of shock and its morbidities in order to improve survival. In the case of trauma, many problems are centered on uncontrolled bleeding, patient instability, and hemorrhagic shock. Major classifications of patient diagnostics can be considered as physical or metabolic.

Physical or global vital signs are at the organismal (whole body) level and include variables such as blood volume, hematocrit, blood pressure, heart rate, body temperature, fluid output (urine), and other physically relevant observations, e.g. pupil dilation [26]. Physical variables can typically be measured directly with simple instruments or medical devices and visual inspection [13, 27]. Physical vital signs are thus the first to be measured by emergency medical technicians, paramedics, and battlefield medics. Early decisions on trauma management, triage and resuscitation are often based on these physical vital signs [28, 29].

More complex metabolic diagnostics involve monitoring of homeostatic mechanisms by either direct or indirect measurement of metabolic products using laboratory analysis or point of care (POC) testing devices. Metabolic markers are monitored to supplement physical vital signs in order to assess body function, predict
mortality and complications, and determine success of surgical interventions and resuscitation.

The following chapter is a critical review of the methods for determining physiologically-relevant biochemical markers that may serve as indicators of occult bleeding and traumatic injury. Attention is given to advances in biomedical devices and microdevice technology that are relevant to trauma, hemorrhage and hemorrhagic shock diagnosis. Because of the inherent subjectivity in interpreting patient vital signs, there is need for 'smart' sensors and information systems for assessing evolving trauma-related shock on a patient-by-patient basis.

This review identifies the opportunity to develop different varieties of biosensors to collect multivariate physiological data and the information systems to fuse and present that data as useful information in the form of a rapidly-interpretable index of present patient condition that could serve to change patient outcomes. Physiologic status markers and monitoring technologies are important for building confidence in novel diagnostic and prognostic platforms and will be considered simultaneously for effectiveness in their use for the management of trauma. A summary of the diagnostic value and limitations of these physiologic status markers are given in Table 1.1.
Table 1.1 Summary of the diagnostic utility and limitations of physiologic status markers.

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<th>Diagnostic marker</th>
<th>Diagnostic utility</th>
<th>Limitations</th>
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<td>Blood pressure</td>
<td>• Hypotension defined at SBP &lt;90 mmHg • Predictive of mortality beginning as high as 110 mmHg</td>
<td>• Circulatory compensation skews blood pressure measurements • Cannot be used to detect occult hypoxia (tissue hypoxia without hypotension)</td>
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<td>Heart rate</td>
<td>• Tachycardia (rate &gt;100 BPM) indicative of need for fluid resuscitation</td>
<td>• Tachycardia may be absent even after major blood loss in as high as 35% of cases</td>
</tr>
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<td>Hematocrit</td>
<td>• Low admittance hematocrit at arrival to an emergency department implicates the presence of a severe injury</td>
<td>• Usefulness limited to late-stage determination of injury severity • Auto-resuscitation and pre-hospital fluid resuscitation will cause continual variation in hematocrit</td>
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<td>Lactate (blood or tissue)</td>
<td>• Surrogate for detection of hypoxia • Prognostic factor of morbidity and mortality for trauma patients with shock</td>
<td>• Currently only considered useful for treatment of patients in intensive care units • Limited to discrete measurement by laboratory and point of care analysis</td>
</tr>
<tr>
<td>Tissue oxygenation</td>
<td>• Correlates to systolic blood pressure and hypoperfusion •Differentiates reversible from irreversible hemorrhagic shock at late stages of trauma</td>
<td>• Has not been formally or rigorously evaluated for the management of trauma</td>
</tr>
<tr>
<td>Redox status</td>
<td>• Trauma patient improvement correlates with electrochemical response due to reactive oxidative species</td>
<td>• Has not been formally or rigorously evaluated for the management of trauma</td>
</tr>
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<td>Glucose</td>
<td>• Hyperglycemic response is indicative of injury severity • Hypoglycemia is indicative of shock</td>
<td>• Limited to discrete measurement by laboratory and point of care analysis</td>
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<td>Potassium</td>
<td>• Predictive of hemorrhagic hypotension (in pigs)</td>
<td>• Very few clinical studies reporting on hyperkalemia in shock • It is not well known if hyperkalemia occurs in humans following shock</td>
</tr>
<tr>
<td>Base deficit</td>
<td>• Measure of acidosis in a trauma patient</td>
<td>• Poor independent marker for distinguishing survivors from non-survivors of hemorrhagic shock</td>
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1.2 Physical vital signs

1.2.1 Blood Pressure

Systolic blood pressure is one of the Advanced Trauma and Life Support (ATLS) standard vital signs for determining hypotension and hemorrhagic shock [30]. In the case of severe blood loss, trauma patients are immediately taken to the intensive care unit (ICU) by first-responders if found having a systolic blood pressure (SBP) of <90 mmHg [31]. The time exposure to shock is a significant danger to any trauma patient, and successful outcome is heavily dependent upon the depth and total duration of hypoperfusion [21, 32]. However, not all trauma victims, even those in the state of shock, will reach a SBP as low as 90 mmHg [31, 33]. Hypotension and hypoperfusion have been observed to begin at SBP as high as 110 mmHg [34-36].

Monitoring blood pressure is relatively simple and can be done in an automated fashion in the hospital setting using catheters in major blood vessels or the heart. Blood pressure monitoring of trauma victims in the field by auscultation is standard for diagnosing vascular injuries, extent of hemorrhage and shock [27]. There are difficulties when continually measuring blood pressure in moving vehicles such as ambulances and helicopters which may render some measurements unreliable [27, 37, 38]. This is of special concern when in austere environments such as on battlefields and resource poor rural and urban areas that do not allow for much sophisticated and automated equipment [27].

Considering the potential of occult hypoxia combined with circulatory compensation, blood pressure measurement with conventional means is less than ideal for
continual monitoring of trauma victims for hemorrhagic shock. Advanced blood pressure cuffs using programmable systems on a chip (PSOC) have been designed to better automate SBP measurement [39]. Integration of all major components and subsystems onto a single chip is an important step toward advancing wireless and mobile oscillometric diagnostics. A small device footprint is attractive to combat medics who are limited in the total weight each can carry. Having more advanced and accurate blood pressure measurements may make field diagnostics and triage more reliable.

Invasive blood pressure monitoring with implantable biosensors is currently nascent in its stages of development. Inductively powered blood flow sensor microsystems have been shown to be effective at monitoring blood flow and degradation in prosthetic vascular grafts, Figure 1.1 [40]. There is additional need for such sensors in determining vascular graft infection and failure [41]. Biosensor technology based on smart hydrogels combined with piezoresistive pressure transducers can produce

Figure 1.1. (A) Inductively powered blood flow microsensor, with an ASIC and two coils. (B) MEMS pressure sensing element used in the microsensor based on silicon nitride and silicon oxide layers with a piezoresistive silicon nanowire field effect transistor [40].
chemomechanical sensors for determining blood pressure in vivo [42]. Advances in CMOS implementation of a linear voltage regulator will enable powering of an implanted wireless blood pressure biosensor [43]. Grand challenges of biocompatibility and hermeticity have been identified as paramount for long-term implantation of such pressure transducers.

1.2.2 Heart Rate

Heart rate has been a standard physical vital sign for hypovolemic shock for several decades [44]. By the ATLS standards for in-hospital patient care, tachycardia (heart rate > 100 BPM) suggests the need for fluids intervention and bradycardia (heart rate ≈ 60 BPM) suggests patient stability [44]. Heart rate is an easy-to-monitor vital sign using auscultation [45]. Absence of tachycardia in a trauma patient may lead to a false sense of hemodynamic stability which may result in delays of emergent interventions [44]. This is an important consideration when numerous animal studies have shown that tachycardia may be absent even after major blood loss [46-53]. Of 489 hypotensive patients of trauma studied, 35% showed no signs of tachycardia [54]. Thus, using heart rate alone to determine the need for resuscitation is a danger to the trauma patient. Heart rate is better utilized in trauma management when considered concomitantly with other vital signs.
Figure 1.2. (A) Edible microsensor by Proteus Biomedical directly attached to a tablet for ingestion. (B) Wearable health monitor that wirelessly communicates with the edible microsensor to collect physiologic data such as temperature and heart rate [55-57].

Although monitoring of hemorrhagic shock by means of heart rate as an independent variable would not be reasonable, it remains a necessary and useful measurement to all caregivers. Decreases in heart rate will follow circulatory decompensation in the case of severe trauma and usher in the state of irreversible shock. Ingestible integrated circuit microchip sensors along with wearable wireless health monitors are currently under development for the measurement of heart rate within the body, Figure 1.2 [55-57]. Non-contact miniaturized biosensors have been able to reach accuracy with mean errors in detection of respiration of 0.5 beat/min and heart rate of 1 beat/min [58]. Continuous heart rate monitors such as these would lessen the need to use auscultation in the field and enable convenient heart rate measurements during patient transit.
1.3. Metabolic markers of stress

1.3.1 Hematocrit

Hematocrit is a measure of percentage of red blood cell volume to whole blood volume [59]. Normal ranges of hematocrit are about \( \geq 35\% \) for women and \( \geq 39\% \) for men [59]. Patients with penetrating trauma, although not receiving fluid resuscitation en route to the hospital, have been observed to present lower hematocrit levels upon arrival to the ED compared patients without significant injury (no surgery needed) [59]. Decreased hematocrit due to trauma is due to self-compensation of interstitial fluid entering the vascular space and due to any pre-hospital fluid resuscitation [60]. Lower admittance hematocrit or greater rates of decrease in hematocrit after emergency department arrival implicate the presence of a severe injury [59].

![Capacitive hematocrit sensor working at 400 kHz. Temperature drift has a large effect on the effectiveness of the sensor and requires compensatory feedback to account for it.](image)

**Figure 1.3.** Capacitive hematocrit sensor working at 400 kHz. Temperature drift has a large effect on the effectiveness of the sensor and requires compensatory feedback to account for it.
Hematocrit is a good late-stage determinant of injury severity, but may not be good for immediate risk assessment by first responders. Auto-resuscitation will occur within 15 minutes after trauma and hemorrhage [61]. It will not be possible for EMS personnel to know the trauma victim’s initial hematocrit, and much would still be left to qualitative observation to estimate blood loss. Dielectric spectroscopy, through permittivity measurements of blood samples, has been shown to be accurate for measuring hematocrit in a continuous fashion in the clinical setting with high accuracy and low error (5%) [62]. Other sensor designs utilize capacitive measurements to determine hematocrit, wherein capacitance of a sample changes due to changing blood cell count to total blood volume, Figure 1.3 [63]. These methods are useful for continual monitoring of hematocrit and patient vital signs, but are very limited to their use in-hospital. Changing hematocrit is also a variable that more often than not confounds readings of glucose and lactate from in-dwelling implantable amperometric biosensors and must be accounted for during design [64].

1.3.2 Lactate

Lactate (lactic acid; pKa = 3.86), is a clinically significant biomarker/metabolite to medical professionals because of its importance in several conditions including; lactic acidosis, severe dehydration, respiratory failure, heart failure, severe infections, alcohol abuse, liver disease, ketoacidosis, hemorrhage and shock. Normal serum lactate is defined by blood values from 0.5 to 2.2 mM for venous blood and 0.5 to 1.6 mM for arterial blood. Hyperlactatemia is considered arterial blood lactate in excess of 2.2 mM, and severely elevated blood lactate levels can be considered greater than or equal to 4
Blood lactate is most often measured via in-hospital laboratory analysis in the lab. Lactic acidosis or hyperlactatemia occurs pursuant to anaerobic metabolism of glucose during tissue hypoxia [67] and is linked to glycogen stores via the Cory cycle. Arterial lactate has been well documented as a predictor of injury severity and a prognostic factor of morbidity for trauma patients with hypovolemic shock [7, 8] and is implicated in patient’s response to vasopressin therapy [68].

Having importance in both the sports and health care applications, continuous lactate monitoring biosensors are under heavy research, design and development. **Figure 1.4.** Some recent novel continuous lactate monitoring transducer designs include implantable needle type [69], implantable multidisc array [70], wearable and flexible graphene-based nano-structured sheets [71], implantable Prussian Blue modified carbon fibers [72], and transdermal microspike arrays [73]. These biosensor systems all have excellent concentration detection ranges of approximately 0.4-7 mM [3], rapid response times of 2-60 s *in vitro* [3, 71] and response times of <10 min *in vivo* [69]. Design of long-term implantable devices requires use of permselective and anti-fouling membranes which can lead to a significant decreases in overall sensitivity; as high as 60-75% [1, 72]. The small footprint of these systems will enable analyte measurement in tissues and environments previously unexplored.
Figure 1.4. Lactate concentration in tissue and blood of a Sprague-Dawley rat. Tissue lactate was continuously monitored with an implantable needle type biosensor and blood lactate was measured using Lactate Pro test strips with venous blood from the tail vein. The implanted biosensor was interrogated by infusing sodium lactate into the blood and the surrounding tissue.

Continual monitoring of lactate may give insight into the dynamic nature of hemorrhagic shock. The temporal behavior and tissue-specific spatial distribution of lactate has not yet been rigorously studied during hemorrhage through the initial onset of hemorrhagic shock. There is clear indication that muscles, being the largest consumer of oxygen, are likewise the dominant source of lactate during hemorrhage. Intramuscular lactate must diffuse into the vasculature in order to establish the familiar venous and arterial lactate values. Interstitial values of intramuscular lactate may therefore be a more appropriate indicator than serum lactate in establishing temporal behavior. Such data may be more useful in determining triage, assessing risk of mortality and co-morbidities, and guiding patient-specific resuscitation strategies. These findings suggest a need to continually monitor the total extent of elevated lactate for determination of total extent of
hypoperfusion and hypoxia rather than taking discrete lactate measurements during trauma management as is currently the standard of practice in hospitals.

1.3.3 Tissue oxygenation

Tissue oxygen saturation (StO₂) is a medical parameter that quantifies the ratio of oxygenated hemoglobin concentration to total hemoglobin concentration in the microcirculation of tissues and is given as an absolute number or percentage. Hemoglobin oxygen saturation (SpO₂) and partial pressure of oxygen (pO₂) are other measures of oxygenation and pertain primarily to blood. Therefore, StO₂ is primarily a measurement of oxygen supply and consumption at the local tissue level and not a measure of systemic oxygenation. Under standard conditions (T = 37 °C, pH = 7.4 and \( P_{CO_2} = 40 \) mm Hg) the partial oxygen pressure at half-saturation of hemoglobin (p50) was 25.7 ± 0.35 mmHg for humans [74, 75], 30.0 (±1.3) mmHg for dogs and 34.1 (±1.8) mmHg for cats [76]. Healthy, ambulatory humans average tissue oxygen saturation of 87% ± 6% measured in the thenar muscle [18]. A cutoff value of <75% StO₂ has been used to discriminate mortality and severe hemorrhagic shock in major blood loss patients [18].
Figure 1.5. NIRS sensor placed on thenar eminence of hand (left). Using the fundamentals of Beer’s law, a spectrum of reflected near-infrared light (700-1000 nm) is used to measure the percentage of hemoglobin saturation (right) [77].

Near-infrared spectroscopy (NIRS) is a technology that has been developed to estimate local tissue oxygen saturation [78]. The technology continually monitors light transmission of cytochrome $aa3$, myoglobin, and hemoglobin based on Beer’s law [78]. Absorption spectra of oxygenated and deoxygenated hemoglobin differ, which allows their relative concentrations to be measured within tissue [78]. The measurements of NIRS are indicative of local venous and arterial oxyhemoglobin concentrations, as well as oxygen delivery and consumption of the sampled tissue bed [78-80]. Cytochrome $aa3$ relates to oxygen consumption by mitochondria [81]. Being a completely non-invasive platform is a major advantage for NIRS. Measurements are typically taken from the thenar eminence of the hand, Figure 1.5 [77].

It has been surmised that tissue oxygen saturation (StO$_2$) is a marker of hypoperfusion that can be used for continual monitoring from the onset of a traumatic injury in order to better identify patients of shock [18]. StO$_2$ of rat skeletal muscle measured by NIRS has been shown to differ between reversible and non-reversible shock arising from severe hemorrhage (ca. 40% TBV) [82]. The NIRS system has been non-
rigorously tested in a battlefield Combat Support Hospital in Tikrit, Iraq in 2005 [77]. Among the cases where it was used, StO$_2$ measurements were reasonably correlated to patient status and were shown to be able to serve as a non-invasive tool for monitoring hypoperfusion and resuscitation in severely injured trauma patients, **Figure 1.6** [77].

![Figure 1.6](image)

**Figure 1.6.** Tissue oxygenation monitored with NIRS immediately prior to and following fluid resuscitation of a warfighter. StO$_2$ closely follows systolic blood pressure in real time (n = 1) [77].

Voltammetry using carbon paste electrodes (CPEs) has also been shown to be an effective electrochemical technique for monitoring tissue oxygenation (oxygen concentration) both *in vitro* and *in vivo* by studying oxygen reduction at the surface of the electrodes [83]. At a potential of -650 mV vs SCE, Sensitivity of -1.49 (±0.01) nA/µM with low limits of detection approaching 0.1 µM have been reported with additional advantages of having no dependence upon pH, temperature, ion concentrations and fluid convection [83]. Needle type oxygen microsensors with wireless telemetry have likewise been shown to be highly stable during short-term implantation (ca. 24 hr) in conscious rats; achieving a sensitivity of 213 (±2) pA/µM [84].
The number of times that tissue oxygenation has been evaluated for management of trauma is too few to make formal recommendations. Further testing is still required to determine if any muscle body is sensitive enough to detect minor changes in perfusion hemostasis. The hurdles to overcome will be tissue site specific probe design, algorithm development and optical modeling [85]. Concerning decision making algorithms for patient resuscitation, the use of NIRS has not shown a significant influence in guiding fluid management, for example, in colorectal surgery patients [86]. There are currently very few recently published prospective studies examining the use of NIRS or tissue oxygenation in trauma victim triage and fluid resuscitation. Further testing to establish the usefulness of this vital sign in early shock management is necessary [32].

1.3.4 Redox status

The redox status of a trauma victim is determined by the milieu of reactive oxidative species that result from trauma [87]. Hemorrhagic shock leads to increases in extracellular hydrogen peroxide, superoxides, and peroxynitrite [87]. Monitoring of the tissue-specific redox status has remained largely unexplored until recently. Electrochemical methods such as cyclic voltammetry have been employed in-vivo to monitor serum redox status after hemorrhagic shock [87]. Preliminary results show that real time improvements can be observed in a recovering trauma patient by monitoring the changes and decreases in peak potentials of cyclic voltammograms [87]. These results demonstrate the versatility of electrochemical techniques in assisting with patient monitoring. Electrochemical methods are generally easy to implement and micron-sized electrodes and electrochemical cells have been developed [70] along with miniaturized
potentiostats. While generally non-specific, there is still much opportunity to explore redox status in relation to trauma states.

1.3.5 Glucose

Glucose is another important metabolite measured in the hospital by blood analysis. Hyperglycemia (arterial glucose ≥110 mg/dL) after injury has been observed in both diabetic and non-diabetic patients [88, 89]. Early investigations have found that i) initial hyperglycemia typically occurs rapidly after injury and ii) severity of shock, based on blood pressure, can correlate to the magnitude of the hyperglycemic response [6]. Acute blood withdrawal has also been shown to induce the hyperglycemic response followed by significant hypoglycemia in rats [90].

It is clear that trauma and hemorrhage have an effect on blood glucose concentrations, making it an excellent biomarker for continuous monitoring during trauma patient care. Glucose, because of its close biochemical link to lactate via glycolysis, its relation to insulin resistance [91-93], and its physiologic link to lactate via the Cory Cycle, may be a suitable metabolite whose real time and temporal levels may be crucial for the prediction and management of hemorrhagic shock [94]. With early and continuous monitoring of glucose, a patient beginning to present the hyperglycemic response may quickly be resuscitated or triaged before further complications can arise. This sort of treatment entails fast, efficient, and accurate glucose monitoring. Research into diabetes has generated much knowledge on the metabolism of glucose and the means by which it is monitored.
Many different glucose biosensors have been developed, including transcutaneous continual monitoring systems [95], capillary blood glucose test strips [96] and handheld cartridge-based blood gas analyzers [97]. Many of these techniques are still considered inaccurate for critically ill patients according to the Clinical and Laboratory Standards Institute standards [96]. Cartridge based blood gas analyzers are becoming widely adopted for fast turn around and critical care decisions [97]. Pilot studies have shown that subcutaneous glucose monitors have been considered “good” when compared against laboratory measurements using Bland-Altman and Clarke error grid analysis in critically injured patients [98, 99].

Implantable biosensors based on osmotic swelling and electroconductive hydrogels (ECH) are attractive new platforms for simple, rapid, sensitive and continuous monitoring of glucose [1, 11, 12, 100, 101]. Oxygen independence is a major advantage of swelling-based glucose sensitive hydrogels (GSHs) because enzymes are not present within the system. Large magnitudes of swelling pressure have been reported for GSHs with sensitivities of approximately 1750 Pa/mM in buffer [101]. Electroconductive hydrogels have significantly better in vitro performance with respect to pheochromocytoma (PC12) cell and human muscle fibroblasts (RMS13) viability and proliferation compared to gold electrodes and base (non-conductive) hydrogels [102].

Novel biotransducers have been fabricated by coating fully integrated electrochemical cell microdisc electrode array (MDEA) transducers with electroconductive hydrogels containing glucose oxidase [12]. Such biotransducers exhibited good in vitro performance with respect to low limits of detection of 0.02 mM.
and response times of 2-8 seconds. These ECH-modified transducers have the drawback of lower sensitivity compared to non-ECH modified transducers [11, 12]. Additional in vivo experiments on such electroconductive hydrogels are lacking and should be the next point of interest for these biosensor systems. Glucose has not been well studied under conditions of controlled hemorrhage or studied during the onset of hemorrhagic shock. Therefore, these novel continuous glucose monitoring devices will need to be tested under such pathophysiologic conditions in order to prove their usefulness in emergency care.

1.3.6 Potassium

Potassium is an electrolyte measured from blood samples during trauma care. There is evidence that fluid resuscitation, especially with packed red blood cells and plasma products, renders hemorrhagic shock patients predisposed to hyperkalemia [103, 104]. Hyperkalemia is characterized by elevated plasma potassium levels (≥5.5 mmol/L) and elevates the risks of cardiac arrhythmia and stand-still in trauma victims [103, 105]. Within 60 minutes of hemorrhagic hypotension at MAP <40 mmHg, rats surviving hemorrhagic hypotension have shown to have significantly lower concentrations of arterial and venous potassium, 5-6 mmol/L, than non-survivors, 6-9 mmol/L [90]. A rise in serum potassium concentration will accompany the onset of hemorrhagic shock in pigs [103]. Correlations of serum potassium to changes in arterial pH, PCO₂, and SvO₂ support its use as an acute ischemic insult marker in hemorrhagic shock, Figure 1.7 [103].
Figure 1.7. Relationship of the changes in arterial partial pressure of CO$_2$ and mixed venous oxygen saturation (SvO$_2$) to arterial potassium levels during hemorrhage in swine. Arterial potassium has potential of being a marker of acute ischemia [103].

Ions are relatively easy to monitor via potentiometric and impedimetric analysis. Impedance-based, contactless electrophoresis microchips offer detection of potassium ions over a linear range of 0.02-7 mM with detection limits of 2.8 µM [106]. Contactless conductivity measurement of potassium is most advantageous in that working electrodes are insulated from the electrolyte thereby enabling simple construction, preventing electrode degradation and mitigating undesirable crosstalk [107]. The power and scope of microfluidic analytical devices in trauma diagnostics will be largely dependent upon their cost, especially considering the need for continual or rapid monitoring of potassium. Low cost materials such as aluminum and poly(methyl methacrylate) (PMMA) have been employed to great success [106].

However, there are very few additional prospective clinical studies that report on hyperkalemia in shock or trauma, and it is not well known whether hyperkalemia occurs in humans following hemorrhagic shock [103]. Initial large animal studies have shown
promising results with significant differences in both arterial and venous potassium concentrations when comparing survivors and non-survivors of hemorrhagic hypotension. However, additional prospective work will be necessary in the clinical setting to evaluate the usefulness of blood potassium as a surrogate for quantizing states of hemorrhage. Some major challenges facing implantable potassium biosensors will include ensuring selectivity and miniaturization of impedimetric microchip architectures. Traditional amperometric detection, which most enzyme-based biosensors utilize, is not likely to be useful in detection of potassium.

1.3.7 Base deficit

Base deficit (BD) is the measure of additional base that must be added to a liter of blood in order to normalize the pH to a standard physiologic value \([108]\). However, base deficit is often calculated and reported in terms of base excess \([109]\). Thus, the more negative value of calculated base excess indicates a higher base deficit and acidosis. Metabolic acidosis can be assessed with base deficit and other known parameters \([110]\). Normal levels of base deficit range from 2 to -2.0 mmol/L \([111, 112]\). A blood gas analyzer may be used to measure partial pressure of CO\(_2\), pH, and HCO\(_3^-\) and base deficit can be subsequently calculated in mEq/L \([108, 109]\). There have been several studies showing the correlation of BD to injury severity and degree of hemorrhage \([113, 114]\).

Base deficit has been shown to be a poor marker for distinguishing survivors from non-survivors of hemorrhagic shock and trauma \([90, 115]\). In a prospective hemorrhagic shock model, no statistical difference was observed in the BD values of rats surviving hemorrhagic shock compared to non-survivors \([90]\). However, there was a significant
increase in base deficit (more negative values) for rats undergoing shock when compared to un-hemorrhaged rats [90]. Measurements of base deficit combined with other markers of acidosis yield better estimates of survival probability [116]. Continually monitoring base deficit would require monitoring of several different blood analytes simultaneously [110]. Hemoglobin, blood PCO₂, and pH all need to be taken into account in order to determine a value for base deficit [110].

1.4. Advances in technology for monitoring trauma

1.4.1 Laboratory standards in trauma diagnostics

Standard means of blood analyte and biomarker measurement in-hospital is typically made via laboratory analysis [117] performed under the Clinical Laboratory Improvement Amendments (CLIA) program (in the USA). Plasma-based assays can be performed on machines such as the Roche Cobas Integra 400 analyzer (Roche Diagnostics, Indianapolis, IN) and the Vitros 250 analyzer (Ortho Clinical Diagnostics, Rochester, NY) [118]. During critical care of patients, blood samples are periodically taken and placed into special tubes which must be kept in ice [119]. Samples are then transported to a laboratory to be prepared, centrifuged, and pipetted to definite volumes for analysis [119]. Data acquired from analysis is transported to responsible physicians for critical decision making [119]. Temperature, timing, and sample preparation all have potential for introducing errors [119, 120]. In-hospital laboratory blood tests usually take anywhere from forty minutes to four hours starting from when a patient is received at a triage station [117, 119, 120].
1.4.2 Point of care devices

Point of care testing has been gaining popularity due to the desire for rapid monitoring of the numerous metabolic markers of stress [3, 117, 118, 121]. For trauma, this involves sampling and analysis of whole blood using hand-held or bench top devices [120, 121] approved by the FDA and that may be CLIA waived. Point of care devices allow for rapid results, easy sample preparation and processing, small sample volumes, small footprint, multifunctional analysis, and low cost [121]. Much emphasis and work is going toward validating these devices in clinical use as either supplements to or as potential replacements for laboratory testing [98, 99, 117, 121-123].

Whole blood analyzers typically work based on electrochemical or optical methods [120]. Ion selective, potentiometric electrodes are used for detection of metal ions such as calcium, magnesium and potassium [120]. Ion selective electrodes measure the potential generated across a selectively permeable membrane reflecting the activity or chemical potential of the analyte of interest in a test solution with respect to a reference solution with known analyte activity. Substrate selective, amperometric electrodes are used for detection of analytes such as pO$_2$, creatinine, glucose, and lactate [118-120]. Accurate bench-top point of care blood analyzers such as the Vitros 9500 (Ortho-Clinical Diagnostics, Rochester, NY) can yield results in approximately twelve minutes [117]. Hand-held point of care blood analyzers such as the i-STAT (Abbott Diagnostics, Abbott Park, IL) and Lactate Pro LT-1710 (Arkray Inc., Kyoto, Japan) can give results in about a minute [117, 120].
1.4.3 Reliability of point of care monitoring

More recent comparisons of POC monitoring versus laboratory monitoring of analytes such as glucose and lactate have been evaluated [117, 118, 122, 123]. The POC monitoring of finger-tip lactate using a handheld lactate sensor (LT-1710) at the point of emergency department triage has shown to be useful in early detection of lactate levels in septic patients, and time of results acquisition from triage was significantly faster by 151 minutes when compared to whole blood analysis in a central emergency department laboratory [117]. A mean difference of +0.26 mmol/L was observed in comparing the readings from POC-capillary to laboratory-arterial lactate analysis [117]. All capillary lactate readings >3 mmol/L using the POC device were successfully triaged [117]. In the low (≤2.2 mmol/L) to mid (2.3-5 mmol/L) ranges of blood lactate, point of care devices showed reasonable concordance (90-99%) with laboratory measurements of blood lactate, Figure 1.8 [118]. The hand-held Lactate Plus (Nova Biomedical, Waltham, MA) blood lactate sensor estimated higher values than those determined by laboratory measurements [118].
Figure 1.8. Bland-Altman plot: White) Concordance of the handheld point of care device, i-STAT (Abbott), compared to laboratory standard measurements with the Vitros system (Ortho Clinical Diagnostics) when monitoring lactate levels of trauma patients. Black) The Integra system (Roche) is another lab analytical standard used as a control [118].

Blood glucose measurements with POC devices have not been observed to significantly differ from laboratory measurements [122, 123]. Arterial and capillary glucose measurements with the SureStep Pro Hospital Meter (Johnson & Johnson, Milpitas, California) in randomized critically ill patients, including those with diabetes, showed a mean difference of 10 mg/dL when compared to standard laboratory methods of blood glucose measurement [123]. Hematocrit levels ranging from 25-55% are required for accurate POC glucose measurements [123]. Abnormal hematocrit levels affect rheological properties and are believed to prevent sufficient blood plasma from reaching the reaction surface of test strips and cartridges in POC devices [123]. Other interferents known to POC glucose monitors include elevated creatinine and bilirubin levels, high arterial oxygen levels, shock-like states, and pH below physiological levels (acidosis) [123].
Clinicians are cautioned as current point of care technology is not yet perfected to be a total replacement for laboratory measurement of blood analytes. Lab measurements are done as plasma-based assays whereas points of care tests are done with whole blood. Time of blood sample preparation in POC devices is short for convenience sake, but this renders results more susceptible to error from sample differences. Test cartridges and strips used in hand-held monitors will also have variability in their performance due to manufacturing. When comparing POC results to delayed laboratory analysis, the current underestimation of blood lactate levels seen in devices such as the i-STAT can lead to a failure to recognize high risk trauma patients or a false perception of hemodynamic instability.

1.4.4 Wireless vital signs monitoring systems

Wireless vital signs monitoring systems, WVSM (Athena GTX, Inc, Des Moines, IA) are advanced physiologic status monitoring systems intended for use in the theatre of war [124]. The WVSM technology automatically monitors vital signs of blood pressure, pulse rate, blood oxygenation, and heart signals via a 3-lead electrocardiograph [124]. Computer decision support systems integrated with WVSMs provide end-users with expertise and recommendations concerning treatment or diagnosis of injuries and disease [124]. On its own, the device is capable of continual patient monitoring, producing up to 4.5 hours of collected data, wirelessly uploading data onto personal computers, and has a battery life of up to 7 hours [124].

Current patient vital signs and previous trends can be automatically integrated with a primary care facility’s computer networks when patients equipped with the
WVSM are brought into close proximity to a Wi-Fi network. Additional software support enables multiple patient monitoring [124]. The WVSM system incorporates easy to understand color-coded and audible alarm systems to assist caregivers with triage decisions at a glance [124]. The mini-Medic (Athena GTX, Des Moines, IA) is a similar type of device that attaches to the forehead of a patient and measures similar vital signs as the WVSM.

The WVSM and the mini-Medic systems are versatile in their ability to monitor multiple vital signs while simultaneously providing decision support to caregivers based on those vital signs. As a package for use in the field, the WVSM weighs approximately 16 ounces [124]. This light weight design is necessary for use in far-forward areas of operation. Drawbacks of the system include the use of blood pressure cuffs, forehead mounts, and ECG leads which require considerable time to put into place and may be problematic dependent upon the nature and type of injury and its location. Use in the battlefield while under hostile fire will also make WVSM deployment more challenging.

The vital signs measured using the WVSM will certainly be able to detect the presence of hemorrhagic shock within decompensated trauma victims but may have more difficulty with compensated trauma patients in assessing their need for resuscitation or lifesaving interventions (LSI). These devices do not yet incorporate direct monitoring of metabolic analytes. Such non-invasive systems must depend upon the fusion of diverse sources of data, and its efficacy is vested in the sophistication of computer decision support systems to aid in the diagnosis and treatment of injuries. Ideally, the WVSM systems will consider more direct variables such as the fundamental metabolic
biomarkers of physiological stress. Biosensors are capable of monitoring metabolic variables in a minimally invasive manner [4] and have great potential to augment current WVSM technology in order to make a more robust physiologic status monitoring platform [3].

1.4.5 In-vivo biosensors for monitoring of trauma

In-vivo biosensors are special point of care testing platforms upon which biological phenomena may be observed and measured in the most direct and continuous manner possible. The means of sensing and detecting biological phenomena are no different from that which can be accomplished on the lab bench or via the hand-held point of care device. Biosensors can provide convenient and timely data with reasonably sized equipment. A successful biosensor system requires a high degree of selectivity, rapid response, repeatability, stability, limited drift, long shelf-life, limited sample preparation, and reasonable cost per test.

Aside from preliminary and pilot studies, no implantable in vivo sensors have been approved for clinical use in trauma management. Animal trauma models and clinical trials are ongoing. Subcutaneous and intramuscular lactate electrodes have been used to examine interstitial lactate during hemorrhagic shock in rats [3, 9]. Reasonable concordance of interstitial lactate to systemic blood lactate has been observed during the non-shock state; which is consistent with previous literature [125, 126]. During the various stages of hemorrhage, from onset to diagnosis of shock, significant differences in both rate of production and final concentration of lactate have been observed when comparing interstitial to blood lactate [3, 9]. Intramuscular lactate levels have been
shown to increase more rapidly than serum lactate during the onset of hemorrhagic shock, but no intramuscular lactate concentrations were reported due to lack of in vivo calibration, Fig. 1.9 [3].

Figure 1.9. In vivo amperometric response of intramuscularly implanted lactate biosensors (n = 5) and systemic blood lactate levels (n = 5) measured using a YSI Biostat Bioanalyzer during onset of hemorrhagic shock (MAP = 40 mmHg). Intramuscular lactate levels are unknown but shown to increase more rapidly than in blood (Error bars show SE) [3].

Disparities between interstitial and blood lactate levels can be attributed to individual or combined effects of a number of possible events such as changes in diffusion coefficients of analytes due to loss of skeletal muscle contractions, loss of interstitial fluid between muscle cells due to auto-resuscitation, muscle cell swelling due to changes in ion and substrate transport systems in the plasma membrane, and decreased peripheral perfusion due to vasoconstriction [61, 127-130]. Using transcutaneous needle-type lactate biosensors, subcutaneously monitored lactate levels have been shown to be lower compared to lactate levels in the blood in a deep state of hemorrhagic shock [9]. This is consistent with previous studies in rats where lower lactate levels were observed
in skeletal muscle compared to blood during states of hypoxia [125, 126]. Fully implanted needle type lactate sensors have shown to maintain stable short-term performance with high sensitivity in interstitial tissue of anesthetized rats, and it was shown that no changes in tissue lactate levels occur during sodium lactate infusions directly into blood [69].

These preliminary results are promising and reinforce the movement toward minimally invasive physiologic status monitoring of trauma through biosensor and biochip technology. More research to observe the temporal aspects of the metabolic events of trauma within various compartments of the body are necessary to acquire statistical power. One of the major limitations to these implantable devices is the difficulty of in vivo calibration. Calibrations made prior to implantation will not be completely accurate due to background signal and interference from electroactive molecules in the tissue environment. Calibrations performed post-implantation often require infusions of the target analyte into the surrounding tissue which can be a cumbersome and time consuming process. Simplicity and accuracy of in vivo calibration is paramount to producing clinically successful implantable biosensors.

1.5. Applications of biomarkers and technology to trauma management

1.5.1 Application of in vivo biosensors to the standard of care

An in vivo biosensor can be small, minimally invasive, wireless, and be powered inductively, by battery, or in the future, by an implantable biofuel cell. Once implanted, the biosensor can be used for continual monitoring of physiologic stress biomarkers. This will allow for practical dynamic monitoring of biological markers for use in better
quantifying the beginnings, complicating events, and endpoints of trauma. The current standard of care for trauma patients in both the military and civilian context is often qualitative. Trauma standards for treatment of all trauma victims still call for physical vital signs in the face of much literature revealing their shortcomings. This is mostly due to lack of proven alternatives. Emergency medical technicians and battlefield medics need to rely on what they know will work for the time being. New technology and innovation in the field of biomedical microdevices are needed, but, more importantly, existing microchip technology must approach translational research to go beyond what is currently known about trauma physiology. The stigma of invasive monitoring techniques needs to be overcome to make way for in-vivo biosensor technology and advance trauma diagnostic technology further than ever before.

1.5.2 Perspectives on the next generation of diagnostics

The use of an in vivo biosensor system for the continual monitoring of analytes relating to hypoperfusion may better indicate severity, onset, and survivability of trauma induced shock. Two important perceptions undergird the current advances in trauma diagnostic biosensors. First, the physiologic status of a traumatized individual can be elucidated through the use of an easily deployable, minimally-invasive biosensor system that has specificity toward the metabolic markers of hypoperfusion that have been discussed. Second, glucose and lactate have proven to be among the foremost clinically relevant analytes to trauma, hemorrhage, shock, resuscitation, mortality, and infection. These perceptions have successfully led biomedical microdevice research to design and develop enzyme-based, substrate-selective electrode biosensors that detect both glucose
and lactate simultaneously [3, 70, 131-133]. **Figure 1.10** shows the schematic for an inductively powered minimally invasive physiological monitoring biochip for the measurement and monitoring of a suite of analytes that include; glucose, lactate, K\(^+\), VO\(_2\), pH and T (°C). The system seeks to combine the IronIC Patch®, a wearable device to power implanted sensors by means of an inductive link [134] with the PSMBioChip®, a minimally invasive implantable biosensor for hemorrhage monitoring [3, 70, 135] to yield a fully integrated system suitable for civilian and military use [136].

![Figure 1.10. Schematic of a multi-analyte implantable in vivo amperometric biosensor (The PSMBioChip®) that is powered inductively by the IronIC Patch® [134].](image)

Implantation of biosensors can be done subcutaneously or intramuscularly to access new avenues of monitoring interstitial metabolism during trauma. For example, skeletal muscle is greatly influenced by trauma by virtue of having the greatest oxygen consumption rate and lowest blood delivery rate compared to all other organs in the body [3, 137]. The amount and duration of intramuscular glucose and lactate may be better
indicators of survivability during hemorrhage than typical vital measurements such as blood pressure and heart rate.

The unknown relationships between interstitial and systemic analytes must still be reliably established during the various phases of hemorrhage and hemorrhagic shock. This knowledge may open new avenues of trauma diagnostics in the field as well as in the hospital setting. Implantable biosensor design criteria will be more stringent than those of *in vitro* devices due to the hostile environment of the body. Some important performance parameters to consider include response time, enzyme stability, biocompatibility, sensitivity, selectivity of analytes, and *in vivo* calibration [138]. The evaluation of these biosensors *in vivo* will elucidate how these design parameters will need to be improved.

Primary long-term goals include the development and deployment of a technology that may, in a military context, be administered to combatants prior to a high risk mission or be administered by a medic to an injured, fallen combatant prior to evacuation. In a civilian context, such a device may be administered by emergency medical personnel to an accident or trauma victim. This device will allow the continuous monitoring of analytes such as tissue lactate and glucose to augment pre-clinical diagnosis of trauma victims during hemorrhage. This may then enable patient specific resuscitation strategies, stratification of trauma patients, and more efficient use of resources such as in the battlefield or mass casualty setting.
1.6. Conclusions: Current standards of care and microdevice diagnostics in clinical practice

Current laboratory methods are cumbersome and require long periods of time before results can be available to clinicians. Use of point of care devices is quick and results are typically easy to interpret via handheld displays, but many critical vital sign measurements such as glucose and lactate are still discrete and time point dependent. This leaves much to be desired in understanding the dynamics of the analytes under test. Use of *in vivo* biosensors for use in physiological status monitoring can and will offer unique advantages to current ICU diagnostics and point of care devices. However, aside from the current standards of laboratory blood testing and point of care testing, little to no reliance has been given to the advanced implantable diagnostic microdevices and technologies. These devices that measure the clinically relevant markers of physiologic stress still need to be rigorously tested *in vitro* and *in vivo* to show significant usefulness in predicting mortality, morbidity and need for resuscitative intervention before they will be considered for use in the clinical setting.

While the need for continued research in novel diagnostic microdevices is apparent, it is still uncertain what sort of information would currently be relevant or useful to first responders and care givers. It is imperative to know and understand the needs of these care givers to better implement new diagnostic platforms. Among the most important considerations are; i) what patient data and information is currently useful in triaging patients, ii) how patient information and status is currently handled and documented, iii) how patient information translates into critical care decisions, and iv) the
methods of information transfer between caregivers. Combining and consideration of simultaneously acquired vital signs into meaningful and relevant information will be a grand challenge. Stronger correlations between vital signs and trauma/hemorrhage are currently needed; this will require more investigation of these vital signs using the currently developing novel technologies and microdiagnostic devices.
CHAPTER TWO: IMPLANTABLE AMPEROMETRIC BIOSENSORS AND DESIGN OF AN IMPLANTABLE DUAL ANALYTE BIOSENSOR

2.1 Introduction

An implantable enzyme amperometric biosensor is a bioanalytical technology that is intended to measure and often remotely transmit a record of specific molecular-level of a biological analyte within the human body [139]. The basic function is to indwell a tissue and to detect, measure and record the levels of a molecule of interest within those tissues. Being amperometric, the biotransducer generally comprises two or three electrodes: a working electrode rendered specific to the analyte of interest via immobilization of the appropriate enzyme, a counter electrode to support the ensuing current, and a reference electrode to provide a suitable voltage plane against which the interrogating voltage may be referenced. Two electrode biotransducers functionally co-join the counter and reference electrodes. Implantable amperometric biosensors generally use enzymes as the biorecognition biomolecule to enable the detection of biochemicals of interest within the body [140]. Enzymes catalyze chemical reactions of specific substrates, the products of which may then be detected via electrochemical oxidation or reduction that occurs at the surface of the working electrode while under a suitable impressed potential. For amperometry, a potential is impressed at the working electrode where the enzyme is immobilized. Bioimmobilization, a major technical challenge in its own right, is often achieved using a number of different techniques [141], and the current generated by the electrochemical oxidation or reduction of the enzymatic reactants or
products generates a measurable current. The potential applied may be with respect to a
reference electrode with a known redox potential such as a silver/silver chloride
electrode, although silver presents rather unique challenges when used in vivo \cite{142}, or
with respect to a pseudo-reference electrode such as platinum, if the magnitude of current
generated is sufficiently small as to not induce polarization.

Amperometry is an electrochemical technique wherein a fixed voltage is
impressed upon the working electrode thereby generating an initial transient current, \(i(t)\),
related to the activity of redox species at the interface. The current decays to a steady
state value, \(i_{ss}\), that is dependent upon the bulk chemical potential of electroactive
species. For a single analyte at a macro electrode (critical length scale \(>25 \mu \text{m}\)), the
resulting current is given by Cottrel’s equation \cite{143}. At a microelectrode (critical length
scale \(<25 \mu \text{m}\)), the current profile, shown in Figure 2.1, is given by a modified form of
Cottrel’s equation (Equation 2.1). Table A.1 and Table A.2 (Appendix A) provides a
summary of available geometries of implantable amperometric electrodes, their
associated forms of the Cottrel’s equation and schematics of the diffusive mass transport
field associated with each type of electrode.

\[
i(t) = \frac{nFA D_{app}^{1/2} C^*}{2\pi^{1/2} t^{1/2}} + \frac{nFA D_{app} C^*}{2r_0}
\]  

(2.1)
Figure 2.1. Voltage applied to cell begins at $E_1$ where no reaction occurs and is stepped up to $E_2$ causing electrode processes to begin and a current transient ensues. Current, $I$, drops off with time according to the Cottrell equation as substance, $C^*$, must diffuse to the electrode surface in order to react.

The goal in the design of implantable amperometric biosensors is to make the steady-state current, $i_{ss}$, directly proportional to the chemical potential of the \textit{in vivo} analyte that is recognized by the enzyme. The bioanalytical sensitivity [$s = (i_{ss} - i_{bl})/\log C$ vs. $\log(C^*/C^{\circ})$] (where $i_{ss}$ is the steady state current, $i_{bl}$ is the base line current, and $C^{\circ}$ is a standard concentration), linear dynamic range (the range of concentrations over which the response of the biotransducer is linear), and the limit of detection [$3(\text{SD}/s)$] (where SD is the standard deviation of the blank or a measure of system noise) are important figures of merit in assessing bioanalytical performance of the biosensor [144].
2.1.1. Generation I biotransducers and unmediated amperometric response

Oxidoreductase enzymes, such as the FAD-dependent (flavin adenine dinucleotide) glucose oxidase (GOx) (EC 1.1.3.4) and FMN-dependent (flavin mononucleotide) lactate oxidase (LOx) (EC 1.1.3.2.) are suitable candidates for the recognition of important physiologic biomarkers related to diabetes care and management and to hemorrhage-associated trauma care and management, respectively. GOx is a structurally rigid glycoprotein with a molecular weight of ca. 160 kDa [145] and consists of two identical polypeptide sub-units, each containing a FAD redox center. In Generation I biotransducers, these enzymes, in the presence of dissolved molecular oxygen, produce equivalents of hydrogen peroxide that may be oxidized anodically at a potential which is positive (typically ≥0.60 V vs. Ag/AgCl) [146] or cathodically reduced at a potential which may be negative (typically 0.0 vs. Ag/AgCl) [147] relative to the redox potential of hydrogen peroxide.

\[
\beta-\delta\text{-glucose} + \text{GOx/FAD} \rightarrow \delta\text{-glucono-\delta-lactone} + \text{GOx/FADH}_2 \tag{2.2}
\]

\[
\text{GOx/FADH}_2 + \text{O}_2 \rightarrow \text{GOx/FAD} + \text{H}_2\text{O}_2 \tag{2.3}
\]

\[
\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2e^- \tag{2.4}
\]

2.1.2. Bioanalytical performance of amperometric biosensors

Enzyme kinetic parameters may be obtained from the steady state current of the amperometric enzyme biotransducer. A Hill or Lineweaver-Burk analysis using Equation (2.5) allows calculation of the apparent enzyme kinetic parameters.
Here $I_{SS}$ is the steady-state current or response after substrate, $S$, addition, $C^*_S$ is the bulk concentration of substrate and $I_{max}$ is the maximum current or response measured under enzyme saturated substrate conditions. The maximum current, $I_{max}$, and the Michaelis-Menten constant of the system, $K_M$ (mM), may thus be determined, allowing evaluation of the contextual performance of the enzyme relative to some standard condition, such as in solution. Commonly known implantable biosensor systems include short-term indwelling continuous glucose monitoring systems such as Dexcom’s 2006 FDA-approved STS [148] and 2007 FDA-approved STS-7 Continuous Glucose Monitoring System for diabetics [149]. Newer implantable biosensor systems are being designed with intended use in trauma management [3].

2.1.3 Design of a dual analyte monitoring biotransducer

The Electrochemical Cell-on-a-Chip Microdisc Electrode Arrays (MDEA 5037) is a dual analyte electrochemical transducer intended for amperometric and voltammetric biosensor analysis when coated with bioactive hydrogel membranes. The MDEA 5037 was developed in conjunction with ABTECH Scientific, Inc. (Richmond, VA) to address the need for simultaneous monitoring of interstitial glucose and lactate [150]. A prototype dual responsive electrochemical biotransducer has been developed using bioactive electroconductive hydrogels [11, 12, 70, 135, 151]. The MDEA 5037 is a single transducer (chip) with two independently addressable electrochemical cells. The

$$\frac{1}{I_{SS}} = \frac{K_M}{I_{max}} \frac{1}{C^*_S} + \frac{1}{I_{max}}$$ (2.5)
geometric layout and microlithographic fabrication of these three-electrode electrochemical biotransducers have been described in detail elsewhere [135] and the details of the assembly and packaging of the chip or die for implantation into small vertebrate animals has likewise been described in detail elsewhere [70]. Briefly, electrochemical transducers (0.2 cm x 0.4 cm x 0.05 cm) were fabricated from e-gun vapor deposited platinum (100 nm) on an adhesion promoting titanium/tungsten (Ti/W) layer (10 nm) deposited onto an electronics grade borosilicate glass (0.5 mm thick Schott D263). The electrodes were fashioned into two separate three-electrode electrochemical cells and the electrodes were passivated with 0.5 micron thick silicon nitride (Si$_3$N$_4$). The nitride layer was then resist patterned and fluoro-plasma etched to reveal the multiple microdiscs of the working electrode (WEA), the large area counter electrode (CE, 7.3 x $10^{-3}$ cm$^2$) and the shared reference electrode (RE, 7.3 x $10^{-5}$ cm$^2$) that were connected to the five bonding pads, Figure 2.2. Each electrochemical cell possesses 37 recessed microdiscs arranged in a hexagonal packed array for each working electrode array. Each disc has a diameter of $\varphi=50$ microns, with center-to-center separation of 100 microns, for a total working electrode area, WEA = 7.3 x $10^{-4}$ cm$^2$ [135]. In general, biospecificity is conferred to each working electrode of the device by immobilizing a bioreceptor to each working electrode.
Figure 2.2. Microscopic photograph of the MDEA 5037 implantable transducer with two addressable cells/channels (Ch1 & Ch2); each cell has a working electrode array (WEA), counter electrode (CE) and reference electrode (RE).

Microdisc working electrodes promote hemispherical diffusion of analytes to their surface having consequences on limits of detection, sensitivity, dynamic range, and response rate [152]. The MDEA 5037 design was selected based on observed bioanalytical performance of lactate biotransducers tested as a function of microdisc feature size, Table 2.1 [2]. Electrode arrays with disc sizes of $r = 25 \, \mu m$ were shown to have excellent limits of detection while maintaining high sensitivity and linear range. Activation, modification, and derivatization procedures have been developed for hydrogel casting upon MDEA 5037 surfaces, Fig. 2.3 [2]. In this body of work the MDEA 5037 system is characterized via chronoamperometry using a facile and reversible electrochemical probe ferrocene carboxylic acid (FcCOOH), a surrogate redox couple, for what would otherwise be a mediator in a mediated enzyme biosensor reaction. Results were compared to the theoretical steady state current response given by the
Cottrell relationship to evaluate performance of the transducer for use in amperometry.

The effects coating an MDEA 5037 with electroconductive hydrogel was examined using electrochemical impedance spectroscopy.

**Table 2.1:** A comparison of the bioanalytical performance of lactate biotransducers formed from MDEA-Pt-Pt electrodes as a function of microdisc feature size. Membranes are electroconductive hydrogels of poly(2-hydroxyethyl methacrylate) = p(HEMA), PEGLOx = PEGylated lactate oxidase and PPy = poly(pyrrole-co-4(3-pyrrolyl)butyric acid) [2].

<table>
<thead>
<tr>
<th>MDEA (Diameter)</th>
<th>Linear Range (mM)</th>
<th>Sensitivity (μA/mM)</th>
<th>Detection Limit (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDEA 50 Pt-Pt [r=25μm, 5184 discs]</td>
<td>0.025 – 4.0</td>
<td>1.99</td>
<td>0.012</td>
</tr>
<tr>
<td>MDEA 100 Pt-Pt [r=50μm, 1296 discs]</td>
<td>0.025 – 3.0</td>
<td>2.30</td>
<td>0.015</td>
</tr>
<tr>
<td>MDEA 250 Pt-Pt [r=125μm, 207 discs]</td>
<td>0.05 – 3.0</td>
<td>1.21</td>
<td>0.045</td>
</tr>
<tr>
<td>SDE Pt-Pt [r=1800μm, 1 disc]</td>
<td>0.10 – 100.0</td>
<td>0.10</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**Figure 2.3.** Optical micrograph of the MDEA 5037 transducer and schematic illustration of surface activation, modification, and derivatization for preparation of electroconductive hydrogel casting [3].

2.1.4 Systems integration with a dual channel wireless potentiostat

Potentiostat technology of this age is facilitating novel research in the field of biosensors [153-158] and translational research is dependent upon making efficient
potentiostat systems for implantable biosensor devices [3, 158-161]. The potentiostat continues to be a viable frontend instrument for biosensor technology as it allows several electrochemical modes of biotransducer interrogation. Among these are amperometry (constant voltage), voltammetry (a swept voltage) and coulometry (charge integration).

For Implantable amperometric biosensor systems to fulfill their desired functionality in the most efficient way possible, developers must pay special consideration to size (footprint), power management, telemetry capabilities, and signal processing [162]. The implantable potentiostat may be discrete or an application specific integrated circuit (ASIC) and may be fully implanted or of the type where the biotransducer is implanted and the instrumentation is affixed externally to the subject/patient. Implantable systems enable observation of biological phenomena in a more natural, physiologically relevant state wherein subjects are able to move about freely while being monitored. A two channel, two terminal wireless potentiostat system, the 8151 potentiostat by Pinnacle Technology, has previously been considered for use in a minimally invasive biosensor system for the management of hemorrhage associated trauma [2, 3]. In this body of work model electrochemical cells are used to consider the merits and drawbacks of the 8151 potentiostat for use with a novel implantable biotransducer, the MDEA 5037 [70].

2.2 Materials and Methods

2.2.1 Components

The 8100-K1 fixed frequency wireless dual potentiostat system was purchased from Pinnacle Technology (Lawrence, KS). The kit contained the Pinnacle 8151 wireless
dual potentiostat, the voltage programmer, and a receiver base station (model 8106) with USB cables. Software for data acquisition (PAL) was also included in the kit.

2.2.2 Accuracy testing of the Pinnacle 8151 potentiostat system

The accuracy of the 8151 potentiostat was tested using discrete combinations of two models or “dummy” cells of known impedance. These model cells served as a virtual device under test (VDUT) and were applied to the two available channels of the dual potentiostat. The dummy cells consisted of i) a single resistor (R) of 10 MΩ (nominally) as prescribed by the manufacturer, and ii) a parallel resistor-capacitor (RC) network of R = 10 MΩ and capacitor of 1 µF (nominally). The single resistor VDUT models facile electrochemical reactions having large heterogeneous electron transfer rate constants with little or no capacitative contribution at the interrogation voltage. The complex RC cell models real electrochemical cells wherein the double layer capacitance sits in parallel with faradaic reactions [163]. Two identical resistor cells and two identical RC cells were acquired from Pinnacle Technology. Bias potentials of 0.50 V were applied to channel 1 and channel 2 during experimentation. Thirteen discrete configurations of R and RC were tested on the two channels of the 8151 potentiostat and the % error between the two channels was calculated for the response to model R or RC cells in the following manner:

\[
\% \text{ Error} = \left| \frac{i_{\text{ch}_1} - i_{\text{ch}_2}}{i_{\text{ch}_2}} \right| \times 100
\]  

(2.6)
Where $I_{\text{Ch1}}$ and $I_{\text{Ch2}}$ refer to the current response of channel 1 and channel 2 respectively. The telemetry distance of the 8151 potentiostat was determined in a laboratory setting. A resistor of 10 M$\Omega$ was connected to channel 1 of the potentiostat and a bias potential of 0.5 V was applied. The potentiostat was moved away from the base station until a loss of signal was observed.

### 2.2.3 Chemicals and reagents

Dulbecco’s Phosphate Buffered Saline (PBS, pH = 7.2), Pyrrole monomer (reagent grade 98+%), 4-(3-Pyrrolyl)butyric acid (PyBA), Ferrocenecarboxylic acid, 2-hydroxyethyl methacrylate (HEMA), tetra(ethylene glycol) diacrylate (TEGDA, technical grade), tert-Butyl acrylate (t-BA), poly(ethylene glycol) methyl ether methacrylate ($M_n = 950$, PEG(950)MEM), N-[Tris(hydroxymethyl)methyl]acrylamide (HMMA, 93%), 2-(Dimethylamino) ethyl methacrylate (DMAEMA, 98%), the photo-initiator 2,2-Dimethoxy-2-phenylacetophenone (DMPA, 99+%), Benzophenone, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, sodium hydroxide, and sulfuric acid were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). 2-(Methacryloyloxy)ethyl 2-(Trimethylammonio) ethyl phosphate (MPC) was purchased from Tokyo Chemical Industry CO., LTD. The HEMA, methacrylate and diacrylate reagents were passed through an inhibitor removal column (Sigma-Aldrich) for removal of the polymerization inhibitors hydroquinone and monomethyl ether hydroquinone before using them in the preparation of the hydrogel cocktail. Pyrrole monomer was purified by double passage through an alumina silicate column. Solutions were prepared
in deionized water prepared by purifying distilled water through a Milli-Q plus (Millipore Inc) ultrapure water system.

2.2.4 Cleaning of transducers

The MDEA 5037 transducers (ABTECH Scientific, Inc., Richmond, Virginia) were first ultrasonicated in DI-water, IPA, and DI-water for 5 minutes each. Next the transducer was placed in a UV-ozone cleaner (Boekel Industries) and irradiated for 10 minutes followed by 1 minute of ultrasonication in IPA. The transducer was then immersed in PBS buffer, made the working electrode of a three electrode electrochemical cell and was electrochemically cleaned by sweeping the potential between 0 to -1.2 V (vs. Ag/AgCl, 3 M KCl) at 100 mV/s for 40 cycles. Transducers were finally thoroughly rinsed in flowing DI-water before use.

2.2.5 Functionalization and derivatization procedures for hydrogel application

Plasma modification of the cleaned MDEA 5037 transducers to generate hydroxyl groups onto the silicon nitride was performed under vacuum in the presence of water vapor using a Harrick Plasma Cleaner (Harrick Plasma). Following plasma activation the bare platinum microelectrode surface was silanized by incubation in a 0.1 wt% solution of 3-aminopropyl-trimethoxysilane (APTMS) in ethanol for 45 minutes followed by ultrasonication in IPA for 5 minutes. Curing was then performed at 20 minute intervals of 40 °C, 110 °C, and 40 °C. To remove the APTMS from the electrodes, the transducer was immersed in PBS buffer (0.1 M, pH = 7.2), made the working electrode of a three electrode electrochemical cell and was electrochemically cleaned by sweeping the potential between 0 to -1.2 V (vs. Ag/AgCl, 3 M KCl) at 100 mV/s for 40 cycles.
Polypyrrole was deposited potentiostatically at 750 mV vs. Ag/AgCl onto the working electrodes of the MDEA 5037 using an electropolymerization solution consisting of 0.4 M Py with 0.1 M repeat units of polystyrene sulfonate as dopant prepared in DI-water and pH-adjusted to pH=4.75 using 0.1 M NaOH. An electropolymerization charge density of 10 mC/cm$^2$ was applied to produce a seeding layer of polypyrrole for hydrogel attachment. Transducers were then immersed in a 0.01 M benzophenone solution prepared in ethanol, removed and allowed to air dry for several minutes followed by UV irradiation (365 nm) for 5 minutes each on the front and back side of the transducer to initiate hydrogen abstraction and termination of both the polypyrrole and primary amine groups of the transducer with benzophenone.

2.2.6 Preparation of hydrogel monomer cocktails

A 1.5 mol % TEGDA cross-linked electroconductive hydrogel (ECH) monomer cocktail was prepared according to a previously described protocol [131] by mixing twelve monomer constituents in a mole % fashion as detailed in Table 2.2. A 1:1 (v/v) solvent solution of ethylene glycol/water (pH $\approx$ 8) corresponding to 40 % of the volume of the monomer cocktail was then added to the monomer cocktail and stirred overnight under UV-free conditions. Bifunctional monomer of 2-methacryloyloxyethyl-4(3-pyrrolyl)butanate (MPB) was first synthesized using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), $N$-hydroxysulfosuccinimide (sulfo-NHS) chemistry by: i) dissolving PyBA in the solvent solution and reacting this with an equi-molar concentration of EDC for 30 minutes, ii) adding an equi-molar concentration of sulfo-NHS and incubating for 5 minutes, and iii) adding an equi-molar concentration of
AEMA and incubating for 2 hours at room temperature. Immediately following MPB formulation all remaining liquid monomer constituents were mixed into the solution followed by the remaining solid monomer constituents and stirred until thoroughly mixed and clear. The resulting monomer cocktail was sparged with nitrogen and stored at 4 °C until ready for use.

Table 2.2. Hydrogel constituents and composition expressed in mol% and mol% of repeat units where noted, HEMA and n-BA were maintained at a constant 10:3 mol ratio and TEGDA was varied for the hydrogel compositions under test.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Mole fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEMA</td>
<td>59.75</td>
</tr>
<tr>
<td>t-BA</td>
<td>5.00</td>
</tr>
<tr>
<td>Pyrrole</td>
<td>7.50</td>
</tr>
<tr>
<td>MPB – Bi-functional pyrrole monomer</td>
<td>1.50</td>
</tr>
<tr>
<td>TEGDA</td>
<td>1.50</td>
</tr>
<tr>
<td>PEG(950)MEM (mol% of repeat units)</td>
<td>5.00</td>
</tr>
<tr>
<td>MPC</td>
<td>5.00</td>
</tr>
<tr>
<td>HMMA</td>
<td>5.00</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>5.00</td>
</tr>
<tr>
<td>AEMA</td>
<td>1.50</td>
</tr>
<tr>
<td>DMPA</td>
<td>1.00</td>
</tr>
<tr>
<td>SPMA</td>
<td>2.25</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
</tr>
</tbody>
</table>

2.2.7 Hydrogel application to MDEA 5037 transducers

Immediately after cleaning and surface functionalization, 50 µL of the ECH cocktail (Table 2.2) was pipetted onto the surface of the MDEA 5037. Excess monomer cocktail was removed by touching the edge of the transducer to a KimWipe®. The thin film was then crosslinked with UV light for 5 minutes on the front and back of the transducer using a UV cross-linker (CX-2000 Crosslinker, UVP, Upland, California)
under an inert nitrogen atmosphere to yield a hydrogel membrane. Gel-transducers were then placed into a convection oven at 60 °C to anneal for 60 minutes. Following annealing, the transducers were immersed in an electropolymerization solution of 0.2 M Py and 0.2 M PSSA (repeat units) for 1 hour and electropolymerization of pyrrole was then performed at 0.8 V vs. Ag/AgCl for a charge density of 10 or 100 mC/cm² to form an electroconductive hydrogel (ECH). Overoxidation of polypyrrole was performed by sweeping the potential from -0.2 to 1.3 V vs. Ag/AgCl at 100 mV/s for 40 cycles to yield an overoxidized electroconductive hydrogel (OO-ECH).

### 2.2.8 Enzyme immobilization onto MDEA 5037 transducers

Amperometric enzyme biotransducers were prepared by placing electrodes, with or without a hydrogel (non-electroconductive) coating, into a solution of 0.2 M Py and 1 mg/mL of GOx prepared in prepared in Milli-Q® water (pH=6.0). Potentiostatic electropolymerization of pyrrole was initiated using an oxidative potential of 0.75 V (without hydrogel) or 0.80 V (with hydrogel) vs. Ag/AgCl. Enzymes were electrostatically and physically entrapped within the electrodeposited polypyrrole network (PPy-GOx|PtMDEA and PPy-GOx-Gel|PtMDEA). The electropolymerized polypyrrole and entrapped enzymes within the hydrogel formed the ECH. The polypyrrole was subsequently overoxidized as previously described.

### 2.2.9 Impedance spectroscopy of MDEA 5037s and VDUTs

Electrochemical impedance spectroscopy was performed using a Princeton Applied Research Potentiostat/Galvanostat model 283 (Princeton Applied Research) coupled to a Solartron 1260 Frequency Response Analyzer (Solartron Analytical).
Transducers were studied in 1.0 mM FeCOOH in PBS buffer at pH = 7.2 at room temperature in three electrode mode (RE: Ag/AgCl, CE: Platinum mesh) and using a sine wave with 10 mV amplitude. Electrical impedance spectroscopy was similarly performed in two electrode mode to characterize the R and RC dummy cells. MATLAB 2011b software was utilized with default Savitzky-Golay smoothing filters for collected spectroscopy data.

2.2.10 Interrogation of MDEA 5037-Pinnacle 8151 wireless biosensor system

A Dell Latitude laptop with PAL software (Pinnacle Technology, Inc.) was used for data acquisition. The bioactive MDEA 5037 was interfaced with the 8151 wireless potentiostat via a custom connector. For the MDEA 5037, two electrode electrochemical detection of glucose was utilized with the on-board working electrode array serving as the working electrode and the on-board counter electrode serving as the shorted counter and reference. Bias potentials were programmed into the 8151 anywhere from 0.4 to 0.65 V and applied to the working electrode array of the MDEA 5037 with respect to the onboard counter electrode. Experiments were performed in PBS (pH = 7.2) at room temperature under gentle stirring.

2.2.11 Electrochemical characterization

Electrochemical experiments were performed using a BAS-100B/W Electrochemical Analyzer with a BAS PA-1 preamplifier module used to amplify the current and to filter out noise (BAS, West Lafayette, Indiana, USA). Chronoamperometric measurements were taken with both MDEA 5037 electrochemical cells simultaneously in a two electrode configuration of on-board working electrode array
vs. on-board counter electrode at a bias potential of 700 mV. Current was observed as a function of FcCOOH concentration in PBS buffer at room temperature (25 °C). Electrochemical impedance spectroscopy experiments were carried out in 1 mM FcCOOH in PBS buffer a three-electrode setup with a working electrode array of the MDEA 5037 as the working electrode, a Ag/AgCl (3 M KCl) reference electrode (RE803; ABTECH Scientific, Inc., Richmond, Virginia, USA) and a platinum mesh counter electrode. A sine wave with 10 mV amplitude was utilized and frequencies of 1 – 10^6 Hz were examined. MATLAB 2011b software was utilized with default Savitzky-Golay smoothing filters for collected spectroscopy data.

2.3 Results and Discussion

2.3.1 Foot print

The 8151 potentiostat can capture signals coming from biotransducers and wirelessly transmit two digitized signals on a 916 Hz band to a central base station that is interfaced to a computer via USB. Transmission may be maintained for up to 72 hours straight using a 3 V lithium battery. The full system with battery weighs approximately 8.7 grams with a footprint of approximately 8.2 mm^3. The device is sufficiently small enough to allow continuous measurement with an implanted biotransducer while maintaining a comfortable size and weight for small animals such rats [164, 165] and fish [166, 167]. Having externalized electronics is the current modality for implantable biosensor systems using this potentiostat; much like other commercially available, subcutaneous glucose monitors [168].
2.3.2 Telemetric performance

The 8151 and MDEA 5037 biosensor system, as seen in Figure 2.4, has previously been shown to have the capability to measure and record two analytes simultaneously at two applied potentials [2]. Preliminary studies on the performance of unmodified MDEA 5037 transducers was performed in solutions of 1:1 mixture of 1.0 mM ferrocyanide and 1.0 mM ferricyanide ($E^0 = 0.76$ V vs. Ag/AgCl). Current was observed as a function of concentration at room temperature (25 °C). Electrochemical measurements were taken with both MDEA 5037 electrochemical cells simultaneously in a two electrode configuration of on-board working electrode array vs. on-board counter electrode. Current was shown to monotonically increase as a function of ferri/ferricyanide concentration at oxidative bias potentials of 0.5 (channel 1) and 0.6 V (channel 2), Figure 2.5. The equi-molar, aqueous ferri/ferricyanide couple is an example of a facile electron transfer reaction with large heterogeneous rate constant at platinum. Calibration curves serve as sufficient evidence that current readings can be taken at different concentrations of solution and at different impressed potentials.
Figure 2.4. Image of (A) the Pinnacle 8151 wireless potentiostat showing the two channels with which it can interface with transducers such as (B) the MDEA 5037 implantable biochip with two independently addressable electrochemical cells, each having a working, counter and reference electrodes.

In a laboratory setting, the 8151 potentiostat maintained a steady and unbroken signal as far away as approximately 75 ft (23 m). If a line of sight to the base station was maintained, this distance can be increased to up to 100 ft (30 m) or more. The response time of the potentiostat was found to be between 2-8 seconds when performing
amperometry. The overall performance makes this system advantageous for long-range monitoring of conscious animals in terms of footprint and telemetry performance. For future \textit{in vivo} characterization, the biosensor system under development will have the biotransducer implanted in the trapezius muscle of rats. Tunneling and exteriorization of lead wires will be necessary in order to interface with the potentiostat. Once the device is interfaced, interstitial glucose and lactate levels can be monitored remotely at an acquisition rate of 1 Hz.

\textbf{2.3.3 Accuracy of response of the 8151 potentiostat channels}

The current response of channel 1 and channel 2 to resistor (VDUT) dummy cells averaged 49.9 (±0.1) nA and 49.6 (±0.0) nA (n=4) respectively. The current response of channel 1 and channel 2 to the complex RC cell averaged 51.1 (±0.1) nA and 54.4 (±0.1) nA respectively (n=6). The % error between the two channels was determined to be 6.4 (±0.3) and 0.7 (±0.1) for RC and R model cells respectively, Figure 2.6. A two-tailed t-test indicated a significant difference in the mean % error when comparing the 8151 potentiostat response to R and RC circuits (p ≈ 10^{-8}). These results indicate that the 8151 potentiostat system’s two channels are within a reasonable 1% error for electrochemical redox reactions that are reversible and facile and can make reproducible results when using both channels simultaneously. A high level of reproducibility for this biosensor system has been observed during interrogation with ferrocene monocarboxylic acid (FeCOOH), a well-known redox mediator, which supports these findings [12].
Figure 2.6. The percent error between channels of the Pinnacle Technologies 8151 wireless potentiostat for complex (RC) and simple (R) circuits, 95% confidence intervals are shown.

For complex circuits using the resistor and capacitor in parallel, the 8151 system shows a large margin of error between the two channels. Ideally, the response from both of the channels ought to be identical; much like was seen for the resistors tested. This difference in measurement will be problematic for complex electrochemical reactions such as during dual analyte sensing where both channels must be utilized simultaneously to monitor the activity of analyte using two redox enzymes. We have established that the bias potentials applied by the 8151 to either of the two channels utilize a common reference, that is, the apparent four poles among the two channels are truly three poles. This means that during operation both of the independent counter/reference electrodes of each electrochemical cell share a single counter/reference electrode between the two independent working electrodes.

For clean MDEA 5037 transducers, having a common reference/counter electrode should not engender any significant changes to predicted chronoamperometric responses based on Cottrel’s equation [143]. Current densities would be evenly distributed to both
of the MDEA 5037 working electrodes during operation in simple electrochemical systems. For fully modified MDEA 5037 biotransducers with hydrogels, polypyrrole, and enzymes immobilized onto their microdisc array surfaces, current densities can be expected to be relatively uneven compared to the bare transducer. The design for the MDEA 5037 was with the intent to utilize each of its electrochemical cells (working, counter, and reference electrodes) independently. Being unable to do this will lead to unpredictable behavior between the two cells of the MDEA 5037. The full and independent characterization of all biotransducers fabricated will be necessary during future experimentation in vitro and in vivo when using the combined MDEA 5037 and the 8151 biosensor system. Unfortunately, additional biotransducer variability will now be introduced by the potentiostat system alone, which is less than ideal.

![Figure 2.7](image.png)

**Figure 2.7.** Electrical impedance spectroscopy of virtual device under test dummy cells (n = 4, error bars indicate standard deviation).

The R and RC dummy cells were tested using electrical impedance spectroscopy at frequencies from $10^0 - 10^4$ Hz. A total of four (n=4) spectroscopic analyses were performed on each of the cells, **Figure 2.7.** Dummy cell resistive element values derived from the 8151 potentiostat were determined by dividing the observed current by the
applied voltage. As expected from the Solartron 1260, both of the resistor dummy cells behaved as a pure resistor with an impedance magnitude of $10^7 \, \Omega$. A phase of approximately 0 was observed for the resistors. The complex RC dummy cells showed capacitive phase response (-90°). A classical Randles circuit with a capacitive element was applied to all of the tested dummy cells, Figure 2.8. Resistor cells R1 and R2 showed a charge transfer resistances of 10.47 (±0.19) MΩ and 9.33 (±0.78) MΩ respectively. Both of the RC cells showed very close concordance with one another. Complex cells RC1 and RC2 showed capacitive elements of 1.341 (±0.010) µF and 1.220 (±0.065) µF respectively, which were slightly higher to the expected 1 µF values but fit the model well.

![Figure 2.8](image)

**Figure 2.8.** (Left) Simple Randle’s equivalent circuit model with series resistance ($R_s$), capacitance (C), and polarization resistance ($R_p$) applied to dummy cell VDUTs. (Right) Randle’s equivalent circuit model with a constant phase element in the place of double layer capacitance.

A slightly higher error was observed for the parallel resistive element for RC dummy cells when applying the Randles circuit model. It is known that real electrified interfaces are most aptly modeled by a resistor in series with a capacitor-resistor in parallel [169]. In the case of the RC circuits tested here, the 1 µF capacitor’s resistive contribution is in parallel to the 10 MΩ resistor which negates its contribution at high
frequencies making it appear as though the RC dummy cell is simply a capacitor. Only at low frequencies of $< 10^{-2}$ Hz (approaching DC) do we observe the effects of the 10 MΩ resistor which can clearly be observed with the 8151 potentiostat as seen in Table 2.3. A two-tailed t-test showed that only one of the resistor circuits, R1, as measured by the 8151, was statistically different from the resistance determined by the Solartron. All other resistive element values were not statistically different. These results indicate that the 8151 is a precise instrument but not completely accurate in both channels. High accuracy electrochemistry goes beyond the scope of this wireless potentiostat system. The 8151 is sufficient for performing simple amperometric measurements wherein the current response can be associated with and calibrated to a biomolecular response.

Table 2.3. Statistical comparison of the values of the dummy cells returned by the FRA 1260 compared to the Pinnacle 8151.

<table>
<thead>
<tr>
<th>Dummy Cell (nominal values)</th>
<th>Solatron 1260 (n=4) Equivalent Circuit Values</th>
<th>Model Error</th>
<th>Pinnacle 8151 (n=4)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 (10 MΩ)</td>
<td>10.47 (±0.19) MΩ</td>
<td>3.2 %</td>
<td>9.96 (±0.03) MΩ</td>
<td>p = 0.03</td>
</tr>
<tr>
<td>R2 (10 MΩ)</td>
<td>9.33 (±0.78) MΩ</td>
<td>3.2 %</td>
<td>9.99 (±0.03) MΩ</td>
<td>p = 0.29</td>
</tr>
<tr>
<td>RC1 (10 MΩ, 1.0 µF)</td>
<td>9.58 (±1.16) MΩ, 1.341 (±0.010) µF</td>
<td>7.0 % 2.5 %</td>
<td>9.48 (±0.33) MΩ n/a</td>
<td>p = 0.85</td>
</tr>
<tr>
<td>RC2 (10 MΩ, 1.0 µF)</td>
<td>11.13 (±3.67) MΩ, 1.220 (±0.065) µF</td>
<td>7.6 % 2.6 %</td>
<td>9.48 (±0.31) MΩ n/a</td>
<td>p = 0.32</td>
</tr>
</tbody>
</table>

2.3.4 Amperometric performance of MDEA 5037 transducers

Ferrocene monocarboxylic acid, a well-known reversible redox probe molecule, was used to interrogate cleaned MDEA 5037s using chronoamperometry. The pKa value for FcCOOH is 4.2, the same as that of ascorbic acid which is a well-known in vivo
biosensor interferent [170-172]. Interrogation was performed in PBS at pH = 7.2 with a concentration range of 0.0–0.2 mM FeCOOH. Each electrochemical cell of the MDEA 5037 was interrogated independently and was analyzed (n = 10). A potential of 700 mV was applied to the working electrode with respect to the on-board counter electrode. The dose response curves of the cleaned, bare transducers were observed and compared to theoretical responses based on steady state diffusion limited current, Figure 2.9.

![Figure 2.9](image)

**Figure 2.9.** The theoretical dose response curves of multidisc array (MDEA) and ultra-microdisc (UME) electrodes for comparison with experimental dose response of cleaned/bare MDEA 5037 electrochemical cells (Experimental MDEA 5037, n = 10) interrogated in two electrode mode using a 700 mV bias potential of working versus counter electrodes; Error bars portray 95% confidence intervals.

The dose response curve of freshly cleaned, bare platinum transducers to FeCOOH showed an expected linear response. The sensitivity of bare transducers averaged approximately 391 ±68 nA/mM and $R^2 = 0.9991$. The performance of Cell A (top cell – Ch1, n = 5, average sensitivity = 386 ±54 nA/mM) and Cell B (bottom cell – Ch2, n = 5, sensitivity = 396 ±86 nA/mM) were not significantly different based on a
two-tailed t-test (p = 0.82). We have concluded that for very facile electrochemical reactions (large heterogeneous rate constants), where the device under test behaves as a resistor, the transducer appears quite capable of delivering desired reproducibility. The theoretical steady state response of a microdisc electrode array (MDEA) and single ultramicrodisc electrode (UME) with equal area to the MDEA 5037 had sensitivities of 205 nA/mM and 34 nA/mM respectively. The MDEA 5037 transducer’s had approximately twice the expected theoretical sensitivity for MDEAs and ten times the expected theoretical sensitivity for single UMEs given by the steady state diffusion limited Cottrell equation.

### 2.3.5 Electrochemical impedance spectroscopy of MDEA 5037s

Electrochemical impedance spectroscopy (EIS) was performed on cleaned, ECH, and OO-ECH modified MDEA 5037 transducers, Figure 2.10. Bare MDEA 5037 transducers showed Bode plots with trends that were identical to previously reported EIS measurements [173]. The magnitude of impedance was lower compared to these previous findings but this is likely due to the higher conductivity of the buffer (Dulbecco’s PBS) that was used for interrogation. Transducers modified with freshly prepared ECH had an overall higher impedance for high (>10⁴ Hz) frequencies and an overall lower impedance for low (<10¹) frequencies consistent with increased solution and reduced charge transfer resistance respectively. These results show that the ECH modified electrodes influenced ion incorporation as well as charge transfer. Charge transfer in polypyrrole occurs by transport of polaron and bipolarons along the backbones of the 1-D polymer chains with hopping between chains [174], the density of which are highly dependent upon the nature
of the dopant counter anions used during the polymerization process [175]. At physiologically relevant temperatures (300 K), hopping conduction modeled by Mott’s variable range hopping is the dominant mechanism of charge transport for polypyrrole [176, 177]. Previous findings have shown that bipolaronic (electronic) conduction of polypyrrole within similar hydrogel systems leads to a decrease in impedance, indiscriminate conducting/insulating impressed potential, ca. three orders in magnitude less compared to ionic conductivity in buffer [178].

![Figure 2.10. Electrochemical impedance spectroscopy of MDEA 5037 working electrode arrays in 1 mM FeCOCOOH, PBS buffer with varying surface modifications.

The Randle’s circuit model was also applied to the ECH-MDEA 5037 transducers but with a conducting phase element (CPE) taking the place of the double layer capacitance in order to account for non-ideal capacitance distribution due to surface heterogeneity. The impedance of the CPE is given by (2.7).

\[
Z_{CPE} = \frac{1}{T(j\omega)^P} \tag{2.7}
\]

Where T is the capacitance parameter and P is the ideality factor ranging from 0.5 to 1 that accounts for surface roughness and for which P = 1 equates to a completely flat
surface [179]. Circuit model elements are summarized in Table 2.4. Transducers were tested from frequencies of 1 to \(10^6\) Hz. Fresh application of an electroconductive hydrogel to MDEA 5037 transducers increased the total capacitive response of the system but this difference was not statistically significant (\(p = 0.2\)) when compared to either cleaned or overoxidized ECH transducers. Overoxidized ECH modified transducers had a higher solution resistance but this was not significantly greater (\(p = 0.08\)) when compared to Cleaned and freshly applied ECH modified transducers. The polarization resistance or charge transfer resistance decreased by a factor of five but this difference was also insignificant (\(p = 0.18\)). This effect was negated as shown by the drastic increase in polarization resistance once the polypyrrole was subsequently electrochemically overoxidized indicating formation of a redox inactive membrane. Both Cleaned and ECH transducers fit the Randle’s model well but the OO-ECH transducer did not as can be seen by the higher degree of error in the polarization resistance. This degree of error indicates that OO-ECH transducers cannot be accurately characterized using simplified models.
Table 2.4. Randle’s equivalent circuit model elements for the MDEA 5037 transducers; standard deviation given in parentheses and %error given for model values compared to actual data. PPy-ECH = Polypyrrole Electroconductive Hydrogel; OPPy-ECH = over oxidized polypyrrole electroconductive hydrogel.

<table>
<thead>
<tr>
<th>MDEA</th>
<th>Rs (Ω)</th>
<th>Error%</th>
<th>CPE-T (F, x10^6)</th>
<th>Error%</th>
<th>CPE-P</th>
<th>Error%</th>
<th>Rp (Ω)</th>
<th>Error%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaned</td>
<td>0.39 (0.01)</td>
<td>6.8</td>
<td>5.55 (0.13)</td>
<td>8.6</td>
<td>0.88 (0.01)</td>
<td>1.3</td>
<td>5330 (3013)</td>
<td>4.1</td>
</tr>
<tr>
<td>PPy-ECH</td>
<td>1.05 (0.23)</td>
<td>7.1</td>
<td>38.09 (27.22)</td>
<td>7</td>
<td>0.61 (0.01)</td>
<td>1.6</td>
<td>1076 (36)</td>
<td>.8</td>
</tr>
<tr>
<td>OPPy-ECH</td>
<td>2.58 (1.75)</td>
<td>12.1</td>
<td>12.31 (6.83)</td>
<td>11.2</td>
<td>0.76 (0.16)</td>
<td>2.2</td>
<td>17714 (WEB only)</td>
<td>48.8</td>
</tr>
</tbody>
</table>

The overall increase in impedance for ECH modified transducers can be attributed to both diffusional limitations imparted by the hydrogel [180, 181] as well as the anion screening capability of polypyrrole that has been previously reported [12]. The faradaic component of the FeCOOH reaction with the MDEA transducer is made more facile with the freshly prepared ECH as can be seen in the low frequency domain. The negative phase angle for all transducers indicates a capacitive response. Overoxidizing the polypyrrole removed this effect while limiting ion intercalation showing that it is indeed the conducting electroactive polymer that is responsible for the reduction in impedance magnitude for the low frequency domain. Conducting gels and overoxidized conducting gels were not purely resistive nor purely capacitive; as shown by the phase ranging from -30 to -80 degrees. The significant (p = 0.006) decrease in the ideality factor from 0.88 to 0.61 shows that adding an electroconductive hydrogel with polypyrrole increases the heterogeneity of the transducer surface.

2.3.6 In-vitro biosensor performance

Polypyrrole doped with GOx was polymerized onto working electrode array (connected to channel 1) of the MDEA 5037 at a charge density of 100 mC/cm². Working
electrode array B (connected to channel 2) was left unmodified to compare response of enzymes that may have non-specifically adsorbed during the fabrication process. The biosensor system was first tested with both channels of the 8151 enabled at a bias potential of 0.65 V. Infusion of glucose lead to an increase in amperometric current for the PPy-GOx modified array whereas no current response was noted for the unmodified array, Figure 2.11. The biosensor had a good response time of approximately 20 s and the biosensor performance parameters are summarized in Table 2.5.

The mechanism of transduction for the biosensor is oxidation of hydrogen peroxide generated from the glucose in the presence of oxygen in the reaction catalyzed by glucose oxidase [1]. These results indicate that no discernable electrochemical crosstalk is occurring between the two working electrode arrays of the MDEA 5037 during glucose detection. No measurable current response was generated at channel 2 as was expected for the unmodified electrode array. Therefore, no appreciable contribution to amperometric response of the biosensor system is occurring from non-specifically adsorbed enzymes.
Figure 2.11. Dose response of the biosensor system to glucose with both channels enabled where Ch1 measures from the bioactive working electrode array and Ch2 measures from the unmodified working electrode array of the MDEA 5037; inset shows amperometric response and error bars show the standard deviation of the steady state response.

Table 2.5. Summary of performance parameters for the 8151 and MDEA 5037 wireless biosensor system.

<table>
<thead>
<tr>
<th>Bias potential (V):</th>
<th>0.65</th>
<th>0.65</th>
<th>0.5</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channels enabled</td>
<td>1 &amp;  2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sensitivity (µA cm(^{-2}) mM(^{-1}))</td>
<td>2.2</td>
<td>1.6</td>
<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Maximum current (µA cm(^{-2}))</td>
<td>45.9</td>
<td>32.8</td>
<td>27.1</td>
<td>14.4</td>
</tr>
<tr>
<td>LOD (mM)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>K_Mapp (mM)</td>
<td>9.4</td>
<td>10.3</td>
<td>7.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Linear dynamic range maximum (mM)</td>
<td>11.6</td>
<td>12.6</td>
<td>7.6</td>
<td>7.1</td>
</tr>
</tbody>
</table>

This wireless biosensor system is intended for use as a dual analyte monitoring system to measure clinically relevant interstitial glucose and lactate simultaneously [70]. Independence between the two channels is paramount to accurate detection of vital signs. Channel 1 was tested independently at bias potentials of 0.4, 0.5 and 0.65 V by
disconnecting the unmodified MDEA 5037’s working region B from channel 2 of the 8151 potentiostat. The dose response curves for bioactive channel 1 are shown in Figure 2.12. Disabling channel 2 lead to a decrease in sensitivity from 2.2 \( \mu A \text{ cm}^{-2} \text{ mM}^{-1} \) to 1.6 \( \mu A \text{ cm}^{-2} \text{ mM}^{-1} \) at a bias potential of 0.65V. Enabling amperometric measurement from channel 2 during glucose detection using channel 1 was shown to lead to a percent error of 9.2%, 22.7% and 43.9% in channel 1 at bias potentials of 0.65, 0.5 and 0.4 V respectively, Figure 2.13. No change in the magnitude of amperometric response should occur when connecting/disconnecting the MDEA 5037 to the two channels of the 8151 if the two channels are truly independent of each other. This crosstalk between channels is likely due to the internal potentiostat instrumentation architecture as no detectable enzymatic current response from channel 2 exists.

![Graph showing bias potential dependence of biosensor performance](image)

**Figure 2.12.** Bias potential dependence of biosensor performance where only Ch1 is enabled during interrogation; error bars show the standard deviation of the steady state response.
Figure 2.13. The influence on current response due to measuring amperometric response from both channels simultaneously at a fixed glucose concentration (30 mM); inset shows the % error of Ch1 when Ch2 is enabled.

2.4 Conclusions

The pinnacle 8151 wireless potentiostat was shown to have excellent response time and telemetric distance for small animal testing. The potentiostat can communicate with a base station in a laboratory setting at a distance of up to 100 feet. The battery life is sufficient for continuous monitoring of up to three days and the size/weight of the device makes it an excellent choice for biosensor animal models. The major drawback for the 8151 is its significant percentage of error between its two channels when measuring “real” biosensors. A 6% error was observed between channels 1 and 2 for complex circuits. This inherent error may lead to problems of inaccuracies during enzyme-based amperometric detection of analytes while using the two channels of the 8151
simultaneously. The MDEA 5037 transducer is a chip with two independently addressable electrochemical cells, and could potentially be used with the 8151 potentiostat for performing dual analyte measurements.

Electrochemical impedance spectroscopy of modified MDEA 5037 transducers and VDUT R/RC dummy cells reveal a close approximation to a simple Randle’s circuit model. The two channels of the 8151 share a common reference/counter electrode and therefore require the independent working electrodes of the MDEA 5037 to utilize a common reference/counter electrode, contrary to its design. The 8151 and MDEA 5037 biosensor was shown to be capable of wireless detection of glucose with the potential for dual analyte measurement as no electrochemical crosstalk occurred during interrogation of the biosensor system. However, there was a discernable change in current response when measuring independently from only one channel of the 8151 potentiostat. This raises a concern for the accuracy of the detection of multiple analytes as the two channels are not independent with respect to commercially available potentiostat instrumentation. This issue can be circumvented by careful calibration of devices prior to use. The 8151 dual potentiostat has excellent performance aside from its lack of customizability. The design of the 8151 potentiostat would benefit greatly by including options to allow the use of two independent reference/counter electrodes for the two channels of the potentiostat as well as sample and hold programming.

The MDEA 5037 biotransducer was shown to be highly reproducible with analyzing ferrocene monocarboxylic acid, (FcCOOH), a facile redox probe. The steady state, diffusion limited current produced at the surface of MDEA 5037 showed excellent
sensitivity to FcCOOH. Experimental dose response curves showed higher sensitivity compared to theoretical dose response curves. No differences were shown in performance between the two electrochemical cells of the MDEA 5037. Casting of hydrogels onto a MDEA 5037 transducer surface utilizing APTMS, polypyrrole seeding and hydrogen abstraction with benzophenone was successful. Electrochemical impedance spectroscopy of modified MDEA 5037 transducers reveals a close approximation to a simple Randle’s circuit model.
CHAPTER THREE: FABRICATION AND CHARACTERIZATION OF BIOTRANSDUCERS BASED ON ELECTROPOLYMERIZED POLYPYRROLE AND ELECTROCONDUCTIVE HYDROGELS

3.1 Introduction

3.1.1 Enzyme immobilization techniques for amperometric biosensors

Stability of enzymes is highly dependent on their mode of immobilization. The immobilization technique to localize different enzymes at the surface of multi-analyte electrodes is an engineering challenge in the fabrication of glucose and lactate biotransducers. This challenge is particularly relevant in the fabrication of fully implantable multi-analyte biosensors when the electrode sites are micron dimensions and are adjacent to each other such as in a CMOS device [70]. Various immobilization procedures have been developed for macro-electrodes or single microelectrodes. Among these are physical adsorption [182], enzyme entrapment and encapsulation within polymer membranes with or without covalent tethering that may be spun applied, dipped-coated, sprayed, covalently immobilized using homo- or hetero-bifunctional conjugation agents [2, 183-185], or adsorbed via cross-linking with bifunctional cross-linkers such as glutaraldehyde [186]. None of these additive techniques are however appropriate for closely spaced microelectrodes on a CMOS device.

3.1.2 Polypyrrole for use in fabricating enzyme-based biosensors

The additive placement and entrapment of enzymes on such devices may be achieved by electropolymerization. Electropolymerization produces controllable thin
films of inherently conductive electroactive polymers (CEP) deposited at electrode sites [187]. During formation such polymers entrap counter anions that serve as “dopants”. In the presence of enzymes, which are often negatively charged, electopolymerization leads to formation of an enzyme enriched membrane layer deposited directly on the electrode [188]. Advantageous properties of conductive polymers such as polypyrrole (PPy) include good environmental and thermal stability [189, 190], corrosion protection of metallic electrodes [191], ease of preparation either chemically [192] or electrochemically [193] as a homo-polymer or co-polymer, nano-structuring [194-197] and precise tuning of its electrical conductivity [198]. The amount of PPy electrodeposited onto a metallic or semiconductor electrode can be carefully controlled based on charge density from which the thickness of the CEP can be calculated [199]. Polypyrrole is also known to impart interference suppression for amperometric biosensors [200] and enables control of the hybrid polymer membrane properties. In this work the effectiveness of polypyrrole to act as a screen for interferents is tested with a novel transducer intended for use as a trauma management platform, the Electrochemical-Cell-on-a-Chip Multidisc Electrode Array (MDEA 5037) [70].

3.1.3 Electroconductive hydrogels for engineering the abio-bio interface

Research on soft electrode materials for engineering the abio-bio interface is a growing field of interest for implantable biosensors, bioelectronic devices and next generation bionics [1, 4, 201, 202]. We have previously introduced a class of hybrid biomaterial, the electroconductive hydrogel (ECH) with the potential to address this need [201]. An ECH is a polymeric blend or interpenetrating network that combines the
electrical, redox and optical properties of conducting electroactive polymers with the environmentally responsive properties and biocompatibility of highly hydrated hydrogels. These hybrid biomaterials are capable of merging several attractive physical, chemical and electrochemical properties [102]. The physical and chemical properties of hydrogels can likewise be modified through varying crosslink density [180], inclusion of voids using porogens [203], the incorporation of highly hydrophilic and/or hydrophobic moieties [204] and the inclusion of bioactive components such as hyaluronic acid (HA), phosphorylcholine (PC) and peptides. Both components lend themselves to molecular engineering (tuning) of properties in diverse applications including; artificial muscles, cell-culture and regenerative medicine, electro-stimulated drug release, and biosensors [201, 205]. Importantly, the combination of these two materials and the process of electropolymerization provide a convenient route for the biofabrication of immobilized biomolecules within hydrogels on microfabricated electrodes surfaces [1, 4].

3.1.4 Research aims

3.1.4.1 Polypyrrole charge density dependence of biosensor performance and storage stability

The simultaneous guiding and immobilization of biomolecules via the process of electropolymerization of pyrrole into an electropolymerized polymer membrane as well as within an existing electrode-supported, swollen hydrogel membrane layer is an attractive method for the electrode specific immobilization of glucose oxidase and other enzymes [11, 206, 207]. Polymer thickness and quantity of immobilized enzyme can be controlled via the electropolymerization charge density (mC/cm²) used to create the
PPy/Hydrogel/enzyme composite film. However, such immobilized enzymes rapidly lose their activity due to denaturation and/or leaching [208]. Over time, significant changes to biosensor performance in terms of sensitivity, dynamic range, response time and limits of detection can be expected. The biotransducer performance as a function of electropolymerized polypyrrole charge density (0.1–100 mC/cm²) within hydrogel modified transducers was examined.

Biosensors can be optimized as a function of incorporated hydrogel and polypyrrole content to maximize these performance criteria. The long-term effects of polypyrrole incorporation on biosensor performance is explored. In addition to the materials selection, longevity of biosensor systems is also a function of their manufacturing, packaging, and storage. During storage, a stable environment for maintaining enzyme activity and hydrogel conditioning requires optimization of buffer type, pH, ionic strength and temperature. The effects of storage on the apparent enzyme kinetic parameters of a biosensor systems utilizing hydrogels designed for intramuscular implantation are outlined. The screening capability of the biosensors to the endogenous interferent, ascorbic acid, was examined. Deleterious influence of PPy on an amperometric electrochemical reaction of ferrocene (Fc-Fc⁺), a surrogate redox couple, for what would otherwise be a mediator in a mediated enzyme biosensor reaction was also investigated. Furthermore, the effect of seeding electrodes with polypyrrole doped with polystyrene sulfonic acid (PSSA) on biosensor fabrication and performance was determined.
3.1.4.2 Physico-chemical properties of ECHs as a function of charge density

Bioactive electroconductive hydrogels combining poly(hydroxyethyl methacrylate) (pHEMA) based chemistry with PPy are currently being developed for use in implantable amperometric biosensor systems intended for use as a trauma diagnostic platform [1, 3, 5], in advanced bionic devices [209, 210], and in neural device engineering [211]. The hydrogel component is rendered bioactive by the inclusion of poly(ethylene glycol) (950)methylethermethacrylate (PEGMEM) and 2-(methacryloyloxy)ethyl 2-(trimethylammonio) ethyl phosphate (MPC). Controlling the electrical impedance properties of the bioactive ECHs can be accomplished through modification of the applied electropolymerization charge density of electrochemically polymerized PPy. It has been previously shown that PPy charge density within an ECH has a significant impact on murine pheochromocytoma (PC12) and human muscle fibroblasts (RMS13) cell growth and proliferation [102]. The RMS13 cell line is of particular interest because of its relevance to intramuscular implantation of biosensors used in trauma management. The PC12 cell line is of particular interest because of its relevance to neural implants. Since these attachment dependent cells are capable of sensing and responding to changes in the physicochemical, and possibly electrical, properties of their environment [212, 213], one possible explanation for the ECH induced impact on their growth is that a change in the mechanical properties of the electroconductive hydrogel occurs as a result of increasing the applied charge density of PPy. Furthermore, the elastic modulus is an especially important property of
bioengineered scaffold materials such as hydrogels and can influence the differentiation of cells [214].

In this body of work the correlations among the different materials properties were elaborated; physicochemical, electrical and mechanical, with their influence on the growth and proliferation behavior of attachment dependent cells. The cells; Rhabdomyosarcoma (RMS 13) and pheochromocytoma (PC 12), were grown on electroconductive hydrogels of increasing electropolymerization charge density. The effects of electropolymerization charge density on the UV-Vis characteristics, hydration characteristics, the elastic modulus and the electrical impedance properties of electroconductive hydrogels based on pHEMA and PPy were explored and related to previously reported cell growth and proliferation studies. The goal is to develop user-controlled hybrid biomaterials, such as these biosmart polymers, for engineering the abio-bio interface of next generation implantable biosensors, bioelectronics devices, and for applications in bionics.

3.1.4.3 Enzyme engineering for improved biosensor performance

Enzymes such as glucose oxidase have a number amino acids in their primary structure to which covalent coupling of other functional moieties can be performed. For instance GOx from Aspergillus niger (EC 1.1.3.4, PDB ID 1CF3) has 15 lysine groups per subunit (2 subunits per molecule of GOx) with primary amines available for modification. Modifying enzymes in such a manner will affect the way they are incorporated into and interact with polypyrrole. As a direct means of improving biosensor performance, i.e. increasing biosensor sensitivity and maximum current, lysine groups of
enzymes were functionalized with i) a pyrrole moiety (monomerization) thereby directly incorporating enzymes into the growing polypyrrole, or ii) a sulfonic acid moiety (sulfonization) to make enzymes more effective dopants. Non-covalent and covalent enzyme modifications of monomerization and sulfonization were performed and the effects on biosensor performance were observed.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

Poly(styrene sulfonic acid) was purchased from Polysciences, Inc. Ferric Chloride was purchased from M.G. Chemicals. Dulbecco’s phosphate buffer saline, Benzophenone (reagent grade 99%), Pyrrole monomer (reagent grade 98+%), 4-(3-Pyrrolyl)butyric acid (PyBA), Ferrocenecarboxylic acid (FeCOOH), 3-aminopropyl-trimethoxysilane (APTMS), Glucose oxidase (GOx, E.C. 1.1.3.4 from Aspergillus niger), 2-hydroxyethyl methacrylate (HEMA), tetra(ethylene glycol) diacrylate (TEGDA, technical grade), poly(ethylene glycol)(950)methylethermethacrylate (PEGMEM), N-[Tris(hydroxymethyl)methyl]acrylamide (HMMA, 93%), 2-(Dimethylamino) ethyl methacrylate (DMAEMA, 98%), 2-aminoethyl methacrylate (AEMA, 97%), the photo-initiator 2,2-Dimethoxy-2-phenylacetophenone (DMPA, 99+%), β-D(+)-glucose, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), N-hydroxysuccinimide (NHS), sulfobenzoic acid (SBA) and all other common solvents were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). 2-(Methacryloyloxy)ethyl 2-(trimethylammonio) ethyl phosphate (MPC) was purchased from Tokyo Chemical Industry CO., LTD. Hetero-bifunctional monomer, 2-
methacryloyloxyethyl-4(3-pyrrolyl)butanate (MPB), was synthesized in house from the conjugation of PyBA (acid) and AEMA (amine). The HEMA, methacrylate and diacrylate reagents were passed through an inhibitor removal column (Sigma-Aldrich) for removal of the polymerization inhibitors hydroquinone and monomethyl ether hydroquinone before using them in the preparation of the hydrogel cocktail. Pyrrole monomer was purified by double passage through an alumina silicate column. Solutions were prepared in deionized water prepared by purifying distilled water through a Milli-Q® plus (Millipore Inc.) ultrapure water system. The glucose stock solution of 1.0 M was prepared and allowed to mutarotate overnight.

3.2.2 Characterization of electrodes and enzyme electrodes

Electrochemical experiments were performed using a BAS-100B/W Electrochemical Analyzer with a BAS PA-1 preamplifier module used to amplify the current and to filter out noise (BAS, West Lafayette, Indiana, USA). All experiments were carried out in a three-electrode setup with platinum microelectrodes (ϕ = 25µm or 100µm; BAS) as the working electrode, a Ag/AgCl (3 M KCl) reference electrode (RE803; ABTECH Scientific, Inc., Richmond, Virginia, USA) and a platinum mesh counter electrode (CE).

Microelectrodes were characterized at various stages of fabrication, including cleaned and APTMS surface modified, using the probe 50 mM Fe(CN)$_6^{3-/4-}$ and in ferrocene monocarboxylic acid (FeCOOH). Before use, enzyme-modified microelectrodes were over-oxidized (OO-PPy) by repeatedly cycling the electrode in PBS (100 mM, pH 7.0) between 0 to 1.2 V vs. Ag/AgCl, 3M KCl for 40 cycles at 100 mV/s.
For immobilized enzyme kinetics, biosensor sensitivity, response time and biosensor stability studies, amperometric measurements were performed at potentials of 0.4 – 0.65 V vs. Ag/AgCl, 3 M KCl and by sequential injection of appropriate aliquots of glucose solution into 100 mM PBS (pH 7.2) under continuous stirring to provide for convective transport. Enzyme electrodes were stored in PBS solution at 4 °C over a period up to 18 days. The kinetic parameters were determined using Lineweaver-Burk analysis of biosensor amperometric response every few days during storage periods.

3.2.3 Enzyme monomerization and sulfonization

Pyrrole butyric acid and sulfobenzoic acid were covalently conjugated to lysine groups of glucose oxidase. For monomerizing enzymes a 0.175 M Py, 0.025 M PyBA solution was prepared in DI-Water with pH adjustment to 4.5-5.5 using 1 M NaOH. For sulfonizing enzymes a 0.2 M Py, 0.625 mM SBA solution was prepared in DI-Water (pH ≈ 4-4.5 without needing adjustment). Then 0.625 mM of EDC, the necessary concentration to achieve 10:1 ratio of PyBA:GOx or SBA:GOx conjugation, was incorporated and allowed to incubate for 1 hour at 25 °C to activate the acid groups of PyBA or SBA. Next 0.625 mM of NHS was incorporated and allowed to incubate for 5 min at 25 °C. Lastly 1 mg/mL of GOx was incorporated and allowed to incubate for 24 hours at 4 °C. Enzyme solutions were immediately utilized for electropolymerization after conjugation was completed to form the covalently conjugated polypyrrole systems of PPy(SBA-\textit{con}-GOx) and P(Py-\textit{co}-PyBA-\textit{con}-GOx). Non-covalently conjugated systems did not incorporate EDC or NHS in the electropolymerization solutions to make
the systems of PPy(SBA)-GOx, P(Py-co-PyBA)-GOx. Control systems (PPy-GOx) were prepared with 0.2 M Py and 1 mg/mL GOx in DI-Water.

**3.2.4 Preparation of the enzyme electrodes**

A 3 mol % TEGDA cross-linked standard hydrogel cocktail was prepared according to a previously described recipe and protocol [131] by mixing HEMA, TEGDA, PEG(400)MA, MPC, HMMA, p(NVP), DMAEMA, and DMPA in typical ratio 78:3:5:1:5:2:5:1 mol%. A 1:1 (v/v) solution of ethylene glycol/water corresponding to 20 % of the volume of the monomer cocktail was then added and the mixture stirred overnight under UV-free conditions. Initially, the working platinum microelectrode (BASi, \( \phi = 100\mu m \)) was mechanically polished with 0.05 \( \mu m \) alumina for 10 min and then washed with nanopore water and acetone, respectively, in order to expose a fresh platinum surface. The electrode was then immersed in 0.5 M sulfuric acid, made the working electrode of a three electrode electrochemical cell and was electrochemically cleaned by sweeping the potential between -0.20 to 1.40 V (vs. Ag/AgCl, 3 M KCl) at 100 mV/s until a steady state cyclic voltammogram was obtained. This electrochemical pretreatment was followed by rinsing with nanopore water several times to ensure good repeatability of electrode surfaces. Following cleaning the bare platinum microelectrode surface was silanized by incubation in a 0.1 wt% solution of 3-aminopropyl-trimethoxysilane (APTMS) in anhydrous toluene for one hour followed by thorough rinsing with toluene and water. The electrode surface was subsequently functionalized with acrylate groups by incubation in a solution of 1 mM acryloyl(polyethyleneglycol)-N-hydroxysuccinamide (ACRL-PEG-NHS, MW 400) in 0.1 M HEPES buffer, pH = 8.5
for 2 hours. Functionalization of electrodes was followed by ultrasonication in Milli-Q® water for 5 minutes and drying with ultrahigh purity nitrogen.

Immediately following cleaning, surface modification and PEG-ACRL functionalization, the bare platinum microelectrode was dipped into the 3 mol% TEGDA cross-linked p(HEMA)-based hydrogel light for 5 min using a UV cross-linker (CX-2000 Crosslinker, UVP, Upland cocktail which had been sonicated and sparged with nitrogen. The thin film was then crosslinked with UV, CA, USA) under an inert nitrogen atmosphere to yield a hydrogel membrane that was approximately 5 µm thick.

Electropolymerization of pyrrole was used as the principal means for bioimmobilization of the enzyme on the hydrogel–modified microelectrode [11]. Before electropolymerization of pyrrole, the hydrogel modified electrode was immersed and incubated in 5.0 ml of an aqueous Py (0.4 M) / GOx (1 mg/ml) solution, pH = 6.0, prepared in Milli-Q® water for 1 h to ensure equilibrium between the hydrogel film and the electropolymerization solution. Finally, potentiostatic electropolymerization was commenced by the application of 0.75V vs. Ag/AgCl, 3 M KCl to the Pt W.E. leading to polypyrrole formation within the hydrogel membrane [2] and the concomitant immobilization of GOx within the hydrogel. This produced an enzyme-loaded, electroconductive polymer PPy-hydrogel composite (PPy-GOx-Gel|Pt). The enzyme electrode was subsequently washed with Milli-Q® water and by PBS to remove any unbound GOx from the hydrogel film. Electropolymerization charge densities of 0.1 – 100 mC/cm² were explored.
3.2.5 Electroconductive hydrogel cocktail preparation

A hydrogel cocktail possessing two cross-linkers, TEGDA and MPB, was prepared. Firstly, a hydrogel monomer cocktail containing 1.5 mol % TEGDA was prepared by mixing monomer constituents on a mol% basis (detailed in Table 1) according to a previously described recipe and protocol [131]. Secondly, 1.5 mol % MPB was separately synthesized via EDC, Sulfo-NHS chemistry in a 1:1 (v/v) solution of ethylene glycol/water corresponding to 40 wt% of the monomer cocktail by: i) dissolving PyBA in the ethylene glycol/water (pH ≈ 8) mixed solvent and reacting this with an equi-molar concentration of EDC for 30 minutes, ii) adding an equi-molar concentration of Sulfo-NHS and incubating for a further 5 minutes, and iii) adding an equi-molar concentration of AEMA and incubating for a further 2 hours at room temperature. Immediately following MPB synthesis all remaining liquid monomer constituents were added and mixed into the solvent followed by the remaining solid monomer constituents and the cocktail stirred until thoroughly mixed and clear. The resulting monomer cocktail was gently ultrasonicated, sparged with nitrogen, and stored at 4 °C under UV-free conditions until use.

3.2.6 Fabrication of electroconductive hydrogel modified electrodes and devices

Platinum interdigitated microsensor electrodes (IME 1025.3 M Pt) for impedance measurements, planar indium tin-oxide electrodes (PITO 150) for UV-Vis spectroscopy and planar platinum or planar gold electrodes (PME Pt 118, PME Au 118, PME Au MA) were acquired from Abtech Scientific (Richmond, Virginia, USA). Electrodes were first ultrasonicated in DI-water, isopropyl alcohol (IPA), DI-water for 5 minutes each.
Electrode surfaces were placed into a UV-ozone cleaner (Boekel Industries) and irradiated for 10 minutes followed by a 1 minute ultrasonication in IPA. Plasma modification of the IME devices to generate hydroxyl groups onto the silicon nitride was performed under vacuum in the presence of small amounts of water vapor using a Harrick Plasma Cleaner (Harrick Plasma). Following plasma activation, the bare platinum microelectrode surface was silanized by incubation in a 0.1 wt% solution of APTMS in ethanol for 45 minutes followed by ultrasonication in IPA for 5 minutes. Curing of the silanol was then performed using 20 minute intervals of 40 °C, 110 °C and 40 °C. The transducer was then immersed in PBS buffer (0.1 M, pH = 7.2), made the working electrode of a three electrode electrochemical cell and was cathodically cleaned by sweeping the potential between 0 and -1.2 V (vs. Ag/AgCl, 3 M KCl) at 100 mV/s for 40 cycles.

Polypyrrole was deposited potentiostatically at 850 mV vs. Ag/AgCl onto the electrode fingers using an electropolymerization solution consisting of 0.2 M Py with 0.1 M repeat units of polystyrene sulfonate as dopant prepared in DI-water and pH-adjusted to pH=4.75 using 0.1 M NaOH. An electropolymerization charge density of 10 mC/cm² was applied to produce a seeding layer of polypyrrole. Transducers were then immersed in a 0.01 M benzophenone solution prepared in ethanol, removed and allowed to air dry for several minutes followed by UV irradiation (365 nm) for 5 minutes each on the front and back side of the transducer to initiate hydrogen abstraction and termination of both the polypyrrole and primary amine groups of the transducer with benzophenone [1]. Transducers were then either spin coated with monomer cocktail or dip cast into
monomer cocktail while allowing excess to be removed by dripping and touching the side of transducers to a Kimwipe®. The hydrogel was crosslinked via UV irradiation for 5 minutes. Transducers were placed into a convection oven at 60 °C for 60 minutes to be annealed. Transducers were then allowed to incubate in the electropolymerization solution for 1 hour prior to electropolymerization. Electrodes were then subjected to subsequent chemically induced oxidative polymerization by immersion in 0.01 M ferric chloride for 15 minutes and/or electropolymerization at 800-850 mV vs. Ag/AgCl to generate the polypyrrole based electroconductive hydrogels (PPy-ECH). Charge densities from 10 to 900 mC/cm² were explored.

### 3.2.7 Hydration and swelling properties of hydrogels

Bulk property characterization was performed on the hydrogel to determine: degree of hydration, DoH, density, ρ, and void fraction, ε. Hydrogel disks fashioned using silicone isolators (664206, Grace Biolabs) as casting molds were placed on a clean, hydrophobic (surface modified with octadecyltrichlorosilane), glass microscope slide. 31 μL aliquots of the sonicated and sparged monomer cocktail were pipetted into the isolator wells and a second microscope slide placed over the filled wells. The isolator|slide apparatus was immediately UV irradiated (365 nm, 2.3W/cm²) for 5 minutes resulting in polymerization. The uniform hydrogel disks were removed from the isolators and gradually hydrated to reduce strain-induced cracking by sequential immersion in ethanol|PBS solutions (75:25, 50:50, 25:75 v/v) for a minimum of one hour each. The disks were finally placed in pure PBS, which was exchanged 3-4 more times every 12
hours to extract any remaining, unreacted monomer or solvent. The hydrated disks were stored at room temperature in PBS buffer until use.

Degree of hydration (DoH) was determined by taking hydrated and dehydrated weights of the disks. For dehydration, disks were placed in a desiccator, which maintained a constant humidity and temperature. Dry weight was determined once the weight remained stable over a 24hr period. Degree of hydration was then determined by Equation (3.1). Volume measurements were taken by employing a method, described by Hughes [215], using Archimedes principle. Density was determined using the measured weight and volume of the hydrated disks and void fraction was determined using Equation (3.2) [216].

\[
\text{DoH} (\%) = \frac{M_{HG} - M_{DG}}{M_{HG}} \times 100\% \\
\epsilon = \text{DoH} \left( \frac{\rho_{HG}}{\rho_{solution}} \right) 
\]

Where \( M_i \) is mass of the polymer, \( HG \) is the hydrated hydrogel and \( DG \) is the dehydrated hydrogel. The hydration properties of PPy-ECH films were determined from dimensional changes (z-axis) measured using a thickness profilometer (Tencor Alpha Step 200). The films were prepared on PME 118-Pt (ABTECH Scientific Inc., Richmond, VA) with a fixed area of 0.725cm\(^2\). DoH was determined by taking hydrated and dehydrated dimensions of the films and calculated using Equation (3.4), which results from combining Equation (3.3) with Faraday’s law and a mass balance. Equation (3.3)
assumes uniform, isotropic swelling normal to the PME area and that the volume of mixing between the PPy-ECH and solvent (water) is negligible.

\[
DoH(\%) = \frac{M^{sol}}{M^{Gel} + M^{PPy} + M^{sol}} \times 100\% \quad (3.3)
\]

\[
DoH(\%) = \frac{\rho^{sol} \Delta V}{(wt\%_{Gel}^{Gel-Py}) (\rho V)_{DG}^{Gel-Py} + \frac{Q MW^{PPy}}{nF} + \rho^{sol} \Delta V} \times 100\% \quad (3.4)
\]

Where \( \rho^{sol} \) is the density of the solvent, \( \Delta V \) is the change in volume due to swelling, \( wt\%_{Gel}^{Gel} \) is the weight percent of the UV-polymerized hydrogel in the PPy-ECH composite, \( V \) is volume, \( Q \) is electropolymerization charge density, \( MW^{PPy} \) is the molecular weight of polypyrrole, \( A \) is area of the film, \( n \) (2.25) [217] is the number of electrons per redox event and \( F \) is Faraday's constant.

**3.2.8 Determination of elastic modulus of hydrogels**

The elastic moduli of the electropolymerized PPy-ECH films were measured using a commercial Atomic Force Microscope (MFP3D-BIO-AFM, Asylum Research, Santa Barbara, CA) installed on an optical microscope platform (Nikon eclipse Ti-U, Nikon Instruments Inc., Melville, NY). The measurements employed Olympus TR400-PB cone-tipped cantilevers with a spring constant of 0.09 N/m. The PPy-ECH films were prepared on 1.0 cm x 2.0 cm x 0.05 cm platinum planar metal electrodes (PME 118-Pt; ABTECH Scientific Inc., Richmond, VA, USA) with various charge densities and imaged under immersion conditions within DPBS buffer filled Petri dishes (\( \phi = 50\text{mm} \)).
Each PPy-ECH film was imaged in five separate 25 μm*25 μm areas that produced a total of 500 data points. The quantitative elastic modulus was extracted from the force-displacement data using the Hertz model for a cone tip.

\[
F = \frac{4E}{3(1-\nu^2)}R^2\delta^3
\]  

(3.5)

F = Force (nN) = Cantilever Deflection (nm) x Spring Constant (0.09 nN/nm), E = Elastic Modulus, R = Probe Radius = 2.5 µm, δ = Indentation Depth, ν = Poisson’s Ratio = 0.29 for poly(HEMA) hydrogel [218]. In view of the composite nature of the hybrid polymers, a Poisson’s Ratio = 0.50 was also explored.

3.2.9 UV-Vis and electrical impedance spectroscopy of hydrogels as a function of charge density

For UV-Vis spectroscopy, PITO transducers were cleaned and modified with PPy-ECH composites of varying charge densities and placed within polystyrene cuvettes containing PBS buffer at room temperature (25 °C) and pH = 7.2. Each sample was analyzed by UV–Vis Spectroscopy using a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader X (BioTek Instruments, Inc., Winooski, VT USA). A spectrum analysis was performed from 300 to 900 nm wavelengths to determine the peak absorbance of PPy within the cast ECHs. Electrochemical experiments were performed using a BAS-100B/W Electrochemical Analyzer with a BAS PA-1 preamplifier module used to amplify the current and to filter out noise (BAS, West Lafayette, Indiana, USA).
Electrical impedance spectroscopy was performed using a Solartron 1260 Frequency Response Analyzer (Solartron Analytical). All impedance experiments were carried out in a co-planar, two-electrode mode to measure the impedance between the digits of the interdigitated microsensor electrodes. Devices were studied at RT in 1 mL of PBS buffer at pH = 7.2 using a 10 mV peak-to-peak sine wave over the frequency range of $10^{-1}$-$10^6$ Hz.

### 3.2.10 Cell culture

PC12 and RMS13 cells were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). PC12 cells were cultured in F-12K supplemented with 2.5% fetal bovine serum (FBS) and 15% horse serum (HS), as well as 50 IU/mL penicillin and 50µg/mL streptomycin (Fisher Scientific, Pittsburgh, PA, USA). RMS13 cells were cultured in RPMI supplemented with FBS (10%) and 50 IU/mL penicillin and 50µg/mL streptomycin. Cells were seeded and grown on planar gold electrodes (PME118 or PME MA; ABTECH Scientific, Richmond, VA, USA) that were modified according to: i) Au*; ii) Au*|Gel; (iii) Au*|PPy; (iv) Au*|Gel-P(Py-co-PyBA). Trypsinized cells were stained with trypan blue and the final cell density (in a volume equivalent to that used in the initial cell seeding) determined using a hematocytometer and inverted light microscope. The morphology of cells attached to the electrode surfaces was determined following staining with rhodamine-phalloidin and DAPI subsequent to fixing with 4% paraformaldehyde. A specific cell proliferation assay aimed at confirming the proliferative state, such as 3H-Thy or BrdU, was not performed as cell numbers were in many cases larger than the original seeding numbers confirming cell proliferation.
Imaging of cells for evaluation of morphology was performed using a Nikon confocal upright microscope.

### 3.3 Results and Discussion

#### 3.3.1 Preparation and characterization of microdisc electrodes

Platinum microdisc electrodes were cleaned, surface modified with APTMS (APTMS|Pt) and then chemically functionalized with PEG-ACRL (ACRL-PEG|APTMS|Pt) for the eventual attachment of the bioactive hydrogel membrane layer and subsequent electropolymerization of Py to simultaneously immobilize the enzyme, GOx, within the hydrogel membrane layer. To confirm the adsorption of the silanol layer of primary amines on the microelectrodes, following solvent cleaning and surface modification by APTMS, electrodes were analyzed by cyclic voltammetry in 1:1 50 mM Fe(CN)$_6^{3-/4^-}$. Following electrochemical cleaning in 0.5M H$_2$SO$_4$ by repeated cycling over the range -0.2 to 1.5V (100 mV/s) vs. Ag/AgCl, 3M Cl$^-$, electrodes were again characterized in 1:1 50 mM Fe(CN)$_6^{3-/4^-}$. Figure 3.1 shows the voltammograms that were obtained for $\phi = 25\mu$m) Pt-ME.
Figure 3.1. Cyclic voltammograms of the Pt-ME (ϕ = 25µm) in 10mM PBS at pH 7.0 in the presence of 5mM Fe[CN]$_6^{3-/4-}$ performed at a scan rate of 100 mV/s. A) A direct comparison of an alumina polished electrode (RED) with one electrochemically cleaned in 0.5M H$_2$SO$_4$ by repeated cycling over the range -0.2 to 1.5 V vs. Ag/AgCl, 3M Cl$^-$ at 100 mV/s (BLUE). B) A comparison of electrochemically cleaned electrode (BLUE) with one that was surface modified by APTMS (RED, APTMS|Pt). C) Multiple cycles associated with electrochemical cleaning. D) A comparison of an electrochemically cleaned electrode (BLUE, |Pt) with an electrode that was surface modified with APTMS (RED, APTMS|Pt) and subsequently electrochemically cleaned as in A above.

Panel A of Fig. 3.1 shows that the electrochemical cleaning protocol improves the microelectrode characteristics in Fe[CN]$_6^{3-/4-}$; producing a voltammogram that approaches microelectrode ideality. Panel B shows the result of APTMS silanol surface modification to be a passivated microelectrode with little access of the redox couple to the electrode. Panel C shows the application of 40 cleaning cycles over the range -0.20 to 1.50 V (vs. Ag/AgCl, 3 M KCl) at 100 mV/s until a steady state cyclic voltammogram was obtained. Panel D shows the result of the characterization repeated following the
electrochemical cleaning protocol to return the electrode to a condition similar to the originally cleaned ideal microelectrode behavior.

Figure 3.2 shows the effect of this electrochemical cleaning protocol on the electrochemical behavior of the probe molecules, Fe(CN)$_6^{3/-4-}$ and FcCOOH, at the electrochemically cleaned and as-modified Pt macrodisc electrode Pt-ME ($\phi = 100\mu$m).

Figure 3.2. Cyclic voltammograms of the Pt-ME ($\phi = 100\mu$m) performed at 100 mV/s. A) A comparison of an alumina polished and electrochemically cleaned electrode (BLUE, |Pt) with one that was surface modified with APTMS (RED, APTMS|Pt) as tested in 10mM PBS at pH 7.0 in the presence of 5mM Fe(CN)$_6^{3/-4-}$. B) A comparison of an alumina polished and electrochemically cleaned electrode (BLUE, |Pt) with one that was surface modified with APTMS (RED, APTMS|Pt) as tested in 10mM PBS at pH 7.0 in the presence of 5mM FcCOOH.

Panel A of Fig. 3.2 shows a modest increase in the peak potential of the APTMS surface modified Pt macroelectrode compared to cleaned bare electrode in the Fe(CN)$_6^{3/-4-}$ redox system. At a pH of 7 it is expected that the immobilized amine functional groups of the APTMS chemisorbed layer will carry some fraction of associated positive charges [219]. The electrostatic attraction of APTMS is likely increasing the electrode boundary concentration of the negatively charged Fe(CN)$_6^{3/-4-}$ causing the observed increase in peak oxidation reduction current. The shift in the reduction potential for the Fe(CN)$_6^{3/-4-}$ can be expected for the APTMS coated electrode as it is less likely to transfer electrons to
the higher concentration of negatively charged ferricyanide in conjunction with electrophilic tertiary amine groups at the electrode surface. The system with ferrocene monocarboxylic acid did not show any peak shifts or changes in peak currents, but rather generated similar currents with the APTMS electrode when compared to the bare electrode. Most important however is the implication that on the microelectrode scale the chemisorbed silanaol layer is contiguous and passivating while on the macroelectrode scale the chemisorbed layer lacks the integrity to serve as a passivation layer and is likely discontinuous and establishes that at Pt-ME (\(\phi = 100\mu m\)) redox active molecules can gain access to the macroelectrode surface.

### 3.3.2 Bioanalytical performance of the immobilized GOx biotransducers

Following hydrogel casting, the immobilized enzyme electrodes were prepared by electropolymerization of pyrrole in the presence of GOx leading to entrapment of GOx within the hydrogel and the fabrication of a bioactive, electroconductive hydrogel. The preparation of glucose responsive biotransducers by the immobilization of GOx via electropolymerization of Py is well documented in the literature [132, 200, 220-223]. Electropolymerization to confer enzyme biospecificity to the Pt-supported hydrogel membrane may be accomplished at different values of electropolymerization charge density. It has previously been established that electropolymerization of Py within electrode-supported hydrogels is initiated at the electrode-hydrogel interface and progresses outwards, being more dense at the electrode-hydrogel interface and progressively less towards the hydrogel-solution interface [224]. The amperometric enzyme-based biotransducers are Type 1 (or generation 1) devices that involve the
measurement of the rate of hydrogen peroxide formation that is linked to enzyme activity. Each type of micro-biotransducer was investigated by generating a dose response curve to glucose in aerated buffer solution at RT. Electrodes were then stored in glucose free buffer solution at 4 °C and periodically analyzed as before by reproducing the dose response curve.

Firstly, a comparison was made of the performance of biotransducers prepared using the current hydrogel method (PPy-GOx-Gel|Pt) to that of biotransducers prepared by entrapping enzyme directly to the surface of a cleaned platinum electrode with electropolymerized polypyrrole (PPy-GOx|Pt). Figure 3.3(A) shows the dose-response curves obtained for systems of PPy-GOx|Pt and PPy-GOx-Gel|Pt where the former was prepared by the electropolymerization of Py in the presence of 1.0 mg/ml GOx at a cleaned and unmodified electrode and the latter was prepared by the electropolymerization of Py in the presence of 1.0 mg/ml GOx at a hydrogel-coated (ca. 5.0 µm) electrode. Both biotransducers were prepared using a charge density of 10.0 mC/cm². Biotransducers showed monotonically increasing current responses to infusions of glucose. The chronoamperometric response was stable under gentle stirring conditions with steady state currents being reached and maintained at response times ranging from 10-70 seconds. It is clear that the non-hydrogel system displays a fourfold higher sensitivity (50 pA/mM) compared to the hydrogel modified biotransducer (12 pA/mM). Secondly, the hydrogel modified biotransducer displays a change in slope and thus shows two linear regimes. Secondly, a comparison was made of the performance of biotransducers prepared by the current hydrogel method when using different
electropolymerization charge densities, Figure 3.3(B). The bioanalytical performance of the various transducers studied is summarized in Table 3.1.

**Figure 3.3.** Glucose dose–response curves of biotransducers (ϕ = 100µm) PPy-GOx|Pt (RED) and PPy-GOx-Gel|Pt composite membrane (BLACK). A) A comparison of PPy-GOx|Pt (RED) and PPy-GOx-Gel|Pt at 10 mC/cm² and B) A comparison of PPy-GOx-Gel|Pt at various electropolymerization charges.

**Table 3.1.** Bioanalytical performance of PPy-GOx|Pt and PPy-GOx-Gel|Pt biotransducers as a function of electropolymerization charge density measured after 7 days of storage in PBS 7.2 at 4 °C.

| Charge density (mC/cm²) | PPy-GOx|Pt | PPy-GOx-Gel|Pt |
|-------------------------|------|------|
| 10                      | 0.1  | 1    | 10  | 20  | 100 |
| Sensitivity (nA/mM/cm²) | 596 (1-10 mM) | 39 (1-15 mM) | 281 (1-15 mM) | 146 (1-6 mM) | 84 (1-6 mM) | 45 (1-5 mM) |
| Linear Dynamic Range (mM) | 1 – 10, | 1 – 15, | 1 – 15, | 1 – 6, | 1 – 6, | 1 – 5, |
| Response Time (s)       | 20   | 20   | 50   | 50  | 70  | 2.8 |
| Detection Limit (mM)    | 0.004 | 0.621 | 0.033 | 0.020 | 0.030 | 0.098 |
| K_M (mM)                | 11.65 | 29.2  | 33   | 6   | 4.6  | 0.57 |
| I_max (µA/cm²)          | 12.7  | 1.7   | 13.2 | 1.9 | 0.7  | 0.6 |

Hydrogels of implantable biotransducers are a needed component for conferring their ability to impart biocompatibility through reduction in protein fouling [131],

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reduction to the inflammatory and foreign body responses [225], and reduction to fibrous encapsulation [3]. The response of the freshly prepared (10 mC/cm²) glucose biosensors based on PPy-GOx-Gel|Pt composite was linear over the range 1 to 6 mM, with a sensitivity of 153 nA/mM/cm² and a response time of 25s. The sensitivity of the freshly prepared (10 mC/cm²) glucose biosensor without hydrogel film (PPy-GOx|Pt) was linear in the range 1 to 10 mM with a sensitivity of 637 nA/mM/cm² and a response time of 18s. The presence of the hydrogel improved the linear dynamic range but reduced sensitivity and response time. This could be due to the enzyme loading in the PPy-hydrogel and/or to the diffusion limitation of glucose through the interpenetrating network of PPy within the hydrogel. The observed difference in response time would have no practical impact on performance. Both polypyrrole and the hydrogel are shown to have an effect on enzyme affinity and stability [226]. The apparent Michaelis–Menten constant $K_M$ and the maximum current response, $I_{\text{max}}$, were calculated from the slope and the intercept of the Lineweaver–Burk plots [227, 228]. For PPy-GOx-Gel composite $K_M = 11.4$ mM and $I_{\text{max}} = 3.3 \ \mu\text{A/cm}^2$ and for PPy-GOx composite $K_M = 7.6$ mM and $I_{\text{max}} = 9.1 \ \mu\text{A/cm}^2$. In both cases the value of the $K_M$ was higher than that independently determined using spectrophotometry in solution ($K_M = 6$ mM).
3.3.3 Effect of electropolymerization charge on the response of ECH modified biotransducers

The charge density dependence of electropolymerized PPy on the sensitivity of the biotransducer was examined. After 7 days of storage at 4.0 °C in PBS 7.2 buffer, the sensitivity of 0.1, 1, 10, 20, and 100 mC/cm² current densities used for GOx immobilization onto biotransducers were compared. Biotransducers with charge densities ranging from 0.1-20 mC/cm² were prepared on a platinum microelectrode and the biotransducer with a charge density of 100 mC/cm² was prepared using a microdisc electrode array that has previously been reported [12, 70]. Amperometric responses were normalized to electrode surface area. The linear dynamic range was shown to be influenced by the charge density. Increasing the total charge density decreased the effective linear dynamic range. Improved linear range has previously been attributed to higher porosity of low charge density polypyrrole [229]. Increasing the overall applied charge density led to an increase in the response time of biotransducers. This result was expected as additional polypyrrole will lead to a decrease in permeability of the bioactive membrane [230]. Average pore radius of mesoporous polypyrrole films has been shown to be approximately 17-19 Å [231]. Low charge densities of electropolymerized polypyrrole have been observed to have higher specific capacitances which subsequently decrease with further applied charge density [232]. For polypyrrole systems, higher specific capacitances are attributed to higher porosity networks that increase permeability of electrolytes through the mesopores [233]. Blockage of these nano-features may result due to the overgrowth of polypyrrole [207, 234]. Other findings have shown that
increasing polypyrrole charge density decreases response time of the amperometric biosensor, but no explanation was proposed as to why this may be occurring [230].

We observed an initial rise followed by a decrease in sensitivity with the maximum sensitivity being observed at 1 mC/cm$^2$, **Figure 3.4.** Very similar trends have been observed for cholesterol oxidase and glucose oxidase biotransducers fabricated with overoxidized polypyrrole [230, 235, 236]. Decreases to sensitivity have been attributed to decreases in permeability of hydrogen peroxide as polypyrrole thickness increases [235]. Polypyrrole film morphology is highly dependent upon the electropolymerization charge density [237] and as a result may be having more influence on the activity of the enzymes. Thicker polypyrrole layers also allow more enzymatic reactions to take place closer to the solution/bioactive-membrane interface with subsequent diffusion of hydrogen peroxide into bulk solution rather than to the surface of the transducer [235, 236, 238].

Fructose dehydrogenase immobilized by polypyrrole has also been shown to have a maximum amperometric current response at a charge density of 1 mC/cm$^2$ for fructose biosensors [239]. The inflection of maximum sensitivity for glucose was at a lower PPy charge density or thickness when compared to some other findings [240, 241]. This indicates that PPy generated within the pHEMA hydrogel reaches saturating levels more quickly than PPy deposited directly onto electrodes, consistent with other previously reported findings [224]. Incorporation of the hydrogel diminishes sensitivity by a factor of four and increases the response time by a factor of two. This was shown to be the case at a charge density of 10 mC/cm$^2$ for PPy-GOx electrodes which had a sensitivity of 596
nA/mM/cm² when compared to PPy-GOx-Gel electrodes having a sensitivity of 146 nA/mM/cm², **Table 3.1.** Polypyrrole based enzyme immobilization onto bare electrodes does not typically reach diffusion limiting levels until polymer thickness reaches >250 nm or a charge density of >100 mC/cm² indicating that the hydrogel has an appreciable influence on biosensor performance [238, 242].

The hydrogel utilized was crosslinked with 3 mol% TEGDA, making it a highly tortuous network even prior to the formation of the interpenetrating network of PPy [216]. It has been shown that the void volume and tortuosity increase dramatically by increasing crosslink density of pHEMA hydrogels [181]. This can lead to both decreased diffusivity of glucose and hydrogen peroxide. Loss of enzyme activity due to the immobilization process cannot be ruled out. For the lower charge densities $K_{Mapp}$ equals approximately the Michaelis-Menten coefficients for GOx in solution [243]. The amount of polypyrrole incorporated into the hydrogels may be influencing the bound to free water ratio which can account for the observed trend in $K_{Mapp}$. Bound water has been shown to decrease in similar hydrogel systems with increasing TEGDA crosslink density [216]. We observed that the overall enzyme activity decreased for charge densities >1 mC/cm² but increased in apparent substrate affinity, **Table 3.1**.
Figure 3.4. Trends in the amperometric current density for 5 mM glucose and the apparent Michaelis-Menten enzyme affinity constant, $K_{M_{app}}$, as a function of electropolymerization charge density (mC/cm$^2$). The charge density of 100 mC/cm$^2$ was determined using a microelectrode array.

It is clear that optimizations of biosensor performance parameters will require choosing the combination of cross-link density and electropolymerization charge density that achieves the best sensitivity, linear dynamic range and lowest detection limit. For example, the crosslink density (mol % TEGDA) may be decreased to accommodate additional interpenetrating network formation via pyrrole electropolymerization. The systems design of an implantable amperometric biosensor in this body of work has shown to have a predictable properties based on electropolymerization of polypyrrole. One of the major drawbacks is the decreases in sensitivity of PPy-GOx-Gel composites. Even so, there are appreciable added benefits in using a PPy-hydrogel composite for biosensing purposes. The PPy-hydrogel composites have been shown to have superior cell viability in vitro compared to PPy or hydrogel alone [201]. Other studies have shown polypyrrole to be positively biocompatible with central nervous tissues [244] and peripheral nervous
tissues [245] and polypyrrole particles have been shown to not illicit inflammation to peritoneal cells after a 6 week period [246].

### 3.3.4 Ascorbic acid interference and effects of the applied potential

Ascorbic acid (AA) is a negatively charged endogenous interferent that is known to perturb biosensor response \textit{in vivo}. Ascorbic acid, along with other negatively charged, redox-active interferents must be screened in order to prevent their non-specific oxidation. Failure to take this into account may lead to significant errors during \textit{in vivo} sensing. A relevant range of oxidative potentials for the oxidation of hydrogen peroxide was examined using a hydrogel coated, 10 mC/cm$^2$ PPy biotransducer. It was noted that for a clinically relevant concentration of AA [247] only 3-5% of the total current response was contributed by AA at the low concentration of 5 mM glucose. \textbf{Figure 3.5.} Even at the highest applied potential of 700 mV the biotransducer maintained a reasonable screening ability.

\textbf{Figure 3.5.} Glucose dose–response curves of biotransducers ($\phi = 100\mu$m) PPy-GOx |Pt (RED) and PPy-GOx-Gel|Pt composite membrane (BLACK). A) A comparison of PPy-GOx|Pt (RED) and PPy-GOx-Gel|Pt at 10 mC/cm$^2$ and B) A comparison of PPy-GOx-Gel|Pt at various electropolymerization charges.
Polypyrrole is known for its interferent screening capability by anion exclusion after overoxidation thereby preventing the diffusion of negatively charged ascorbic acid, uric acid, and acetaminophenol [200, 248]. Polypyrrole used with similar hydrogel systems have been able to suppress interferents of ascorbic acid and L-cysteine at twice the physiological levels found in serum by maintaining their deviations to less than 5% of a glucose responsive biosensor [249]. Polypyrrole shows a consistency in its screening ability for interferents in bioactive pHEMA-based hydrogel membranes. Thicker polypyrrole layers with less incorporated enzyme will have the drawback of decreased sensitivity and linear range but increased screening capability of interferents [250].

### 3.3.5 Time dependence of performance – stability

Figure 3.6 shows the amperometric sensitivity of two biotransducers (1.0 and 10 mC/cm\(^2\)) that was observed over time. An overall trend of increasing biotransducer sensitivity as a function of storage time was noted. The biotransducer of 1.0 mC/cm\(^2\) plateaued at approximately 21 pA/mM at 15 days of storage. The biotransducer at 10 mC/cm\(^2\) had a similar trend but was not observed beyond 10 days of storage. The most dramatic changes in stability of PPy-GOx biosensors have been shown to occur within the first 5 days of storage [241]. Typically biotransducers are characterized by a decrease in sensitivity and maximum current with increasing storage time [251]. Amperometric biotransducers utilizing PPy have been shown to have 21 days of stability but a very sharp decrease in sensitivity thereafter [208]. Ideally no changes to sensitivity or maximum current should occur over time. Stability of up to 20 days, with no changes in sensitivity, has been observed for PPy-GOx systems using electrochemical surface
plasmon resonance [252]. Given proper calibration, the stability of the polypyrrole based enzyme amperometric biosensors have a reasonably long lifetime of use.

Figure 3.6. Temporal changes in the sensitivity of stored PPy-GOx-Gel|Pt biotransducers prepared with 1 and 10 mC/cm$^2$ as a function of days of storage in PBS 7.2 at 4 °C.

The apparent Michaelis-Menten constant ($K_{\text{Mapp}}$) for the charge density of the electroconductive hydrogel prepared with an electropolymerization charge of 1.0 mC/cm$^2$ was found to fluctuate over time, Table 3.2. For biorecognition membranes prepared with an electropolymerization charge of 10.0 mC/cm$^2$ the apparent Michaelis-Menten constant ($K_{\text{Mapp}}$) did not change as dramatically, Table 3.3. Enzyme stability and affinity appeared to be maintained considerably higher over time for membranes prepared with 10 mC/cm$^2$, indicating that the additional polypyrrole introduced more enzyme and aids in maintenance of enzyme activity. Michaelis constants ranging from 2.7 - 6.1 mM indicate that affinity increases for polypyrrole entrapped GOx compared to native GOx under aerated conditions which typically have $K_{\text{Mapp}}$ of approximately 11 mM [253, 254].
Table 3.2. Bioanalytical performance and apparent enzyme kinetic parameters of a PPy/GOx/Hydrogel|Acrylate-PEG-APTMS|Pt biotransducer following preparation using an electropolymerization charge density, \( Q = 1.0 \) mC/cm\(^2\) and measured at RT after 7, 10, 11, 15, 16 and 18 days of storage in PBS 7.4 at 4 °C.

<table>
<thead>
<tr>
<th>Days stored</th>
<th>7</th>
<th>10</th>
<th>11</th>
<th>15</th>
<th>16</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (nA/mM)</td>
<td>11.6</td>
<td>17.5</td>
<td>18.3</td>
<td>22.1</td>
<td>18.6</td>
<td>21.2</td>
</tr>
<tr>
<td>Linear dynamic range (mM)</td>
<td>1-8</td>
<td>1-10</td>
<td>1-15</td>
<td>1-15</td>
<td>1-15</td>
<td>1-15</td>
</tr>
<tr>
<td>Response time (s)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Apparent Michaelis-Menten constant (mM)</td>
<td>18.6</td>
<td>152</td>
<td>56</td>
<td>33</td>
<td>31.4</td>
<td>104</td>
</tr>
<tr>
<td>Maximum current (nA)</td>
<td>0.29</td>
<td>2.90</td>
<td>1.33</td>
<td>1.04</td>
<td>0.84</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 3.3. Bioanalytical performance and apparent enzyme kinetic parameters of a PPy/GOx/Hydrogel|Acrylate-PEG-APTMS|Pt biotransducer following preparation using an electropolymerization charge density, \( Q = 10.0 \) mC/cm\(^2\) and measured at RT after 3, 4, 7, 8 and 10 days of storage in PBS 7.4 at 4 °C.

<table>
<thead>
<tr>
<th>Days stored</th>
<th>3</th>
<th>4</th>
<th>7</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (pA/mM)</td>
<td>6.4</td>
<td>3.6</td>
<td>8.5</td>
<td>10.6</td>
<td>12.8</td>
</tr>
<tr>
<td>Linear dynamic range (mM)</td>
<td>0-7</td>
<td>0-7</td>
<td>1-10</td>
<td>1-10</td>
<td>1-15</td>
</tr>
<tr>
<td>Response time (s)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Apparent Michaelis-Menten constant (mM)</td>
<td>2.7</td>
<td>2.1</td>
<td>4.9</td>
<td>6.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Maximum current (pA)</td>
<td>45.5</td>
<td>30</td>
<td>100</td>
<td>147</td>
<td>170</td>
</tr>
</tbody>
</table>

It is expected that most of the enzyme molecules that were incorporated through entrapment would somehow have their native conformation and active site perturbed. Changes in protein structure as a result of immobilization may increase or decrease enzyme affinity for substrates. However, it has been shown that substrate specificity of GOx is unaffected by the immobilization through electropolymerization of pyrrole [222].
Polypyrrole has been shown to protect entrapped enzymes from leaching out in some cases as well as protecting and stabilizing their activity [222, 223, 255]. Higher charge density entails a thicker layer of polypyrrole grown upon the electrode surface allowing greater immobilization and stabilization of enzyme resulting in more consistent enzyme affinity. Upon reaching a diffusion limited state, the current response of biosensors become mass transport controlled. This is usually characterized by a decrease in maximum current and sensitivity with increasing film thickness [238]. Assuming a uniform current distribution to the working electrode, potentiostatic growth of polypyrrole films grown at a charge density of 45 mC/cm² onto planar platinum substrates will produce films of 0.1 µm thickness [242]. Thus, the charge densities utilized in this work effectively generate polypyrrole films that are 2.2 nm and 22 nm in thickness for charge densities of 1.0 and 10.0 mC/cm² respectively which are well below the polypyrrole diffusion limiting thickness of 250 nm.

It is uncertain as to why a maturation process occurs for the investigated PPy-GOx-Gel composite biotransducers. One possibility is that hydration of the polypyrrole matrix is occurring over time. The applied potential of 400 mV vs. Ag/AgCl during interrogation is believed to be high enough to induce degradation of polypyrrole [256], however, the polypyrrole of the current biotransducers are already overoxidized. There is the possibility of some partial enzyme unfolding during electro-immobilization that may be accompanied by refolding within the hydrogel during storage leading to more of the entrapped enzymes to become active, increasing the enzyme efficiency of the
biotransducer. Experimental determination of enzyme secondary structure using FT-IR and circular dichroism may be performed in the future to determine if this is the case.

3.3.6 Interrogation of biotransducers with ferrocene monocarboxylic acid

The dose-response of the transducer modified with OO-PPy showed a decreased average sensitivity to FeCOOH with 28 ±31 nA/mM, a reduction of 92%, Figure 3.7. This was significantly different (p = 10^{-6}) when compared to cleaned/bare electrodes which had an average sensitivity to FeCOOH of 350 ±25 nA/mM. The drastic loss of current response is likely due to anion exclusion of the negatively charged FeCOO^- (as the anionic form of FeCOOH, pKa = 4.2) [257] is electrostatically repulsed by dopant anions within the positively charged OO-PPy film. When comparing the sensitivities of the bare electrode response to the polypyrrole coated electrode response, this ratio is approximately 12:1. This represents effective interference shielding (a rejection ratio of 12:1) of anionic interferents, such as ascorbic acid, that plague amperometric biosensors. These results can be confirmed through amperometric response of OO-PPy transducers to FeCOOH as a function of buffer pH that result in shifts of anionic to neutral forms of FeCOOH.
**Figure 3.7.** The dose response of cleaned/bare and OO-PPy MDEA 5037 electrochemical cells (for both populations n=4) interrogated in two electrode mode using a 700 mV bias potential of working versus counter electrodes; Error bars portray 95% confidence intervals.

**3.3.7 UV-Vis characterization of PPy-ECH composites as a function of charge density**

To confirm and monitor electropolymerization of PPy within the bioactive electroconductive hydrogel films, spectrum analysis was performed from 300 to 900 nm wavelength for all samples, Figure 3.8. Increasing electropolymerization charge density led to an increase in absorbance over the wavelengths range. Very low charge densities (<20 mC/cm$^2$) had very similar absorbance spectra. Limited discrimination at these low charge densities within the PPy-ECH arises because of the presence of the polypyrrole seeding layer needed for the covalent attachment of the PPy-ECH film to the transducer. Polypyrrole absorbs broadly with peaks at wavelengths of ca. 435 nm ($E_g = 2.81$ eV) and 900 nm [258] respectively, and this was also observed for the PPy-ECH|PITOEs for all charge densities. Peak absorbance was taken at these values for each charge density, Figure 3.8 insert. Absorbance increased linearly with increasing charge density within
the PPy-ECH having a linear correlation coefficient of 0.98 for both the 430 and 900 nm wavelengths. This confirms that the PPy-ECH composite may be fabricated with fine control and predictability of its composition and that its optical absorbance can be tuned via electropolymerization. Furthermore, the linearity of the dependence implies a uniform distribution of the PPy within the hydrogel matrix.

![Figure 3.8](image_url)  
**Figure 3.8.** Absorbance spectra of PPy-ECH modified PITOE s at varying charge densities of electropolymerized polypyrrole measured in PBS buffer (pH = 7.2). Insert. Absorbance values at 430 nm and 900 nm as a function of applied charge density in the electropolymerization of pyrrole to form the PPy-ECH.

### 3.3.8 Hydration properties of Py-hydrogel and PPy-ECH composites

Parameters of percent hydration, void fraction and density for freshly cast, UV cross-linked hydrogel, with and without electropolymerized polypyrrole, were determined, **Figure 3.9.** The density of the hydrogel was found to be 1.11 (±0.09) g cm\(^{-3}\), slightly higher than that of water. The degree of hydration for the as-cast hydrogel averaged 68.6 (±0.8)% (n=3) and had a void fraction of 76.4 (±6.8)%. For the freshly
cast, UV cross-linked hydrogel, the total crosslink density was 1.5 mol%, established by the presence of TEGDA. The degree of hydration of the free-standing hydrogel disc measured by the standard gravimetric technique was compared to that for the electrode-supported hydrogel measured using the thickness profilometer (66.4%) and was found to be in good agreement. The observed degree of hydration as a function of crosslink density was similar to values that were previously reported [216]. The void fraction was very high when compared with non-pyrrole containing pHEMA hydrogels previously characterized that had void fractions ranging from 25-75% [12, 216]. Hydrogels based on alginates have been shown to have void fractions ranging from 45-78% [259, 260]. Overall, these hydration and void fraction characteristics are consistent with the incorporation of and hosting of biomolecules such as drugs, peptides, proteins and enzymes [216, 261]. Biomolecule immobilization can be performed by direct incorporation into the monomer cocktail [262], specifically through the covalent attachment to the backbone of the hydrogel via conjugation to free pendant primary amine or carboxylic acid groups [263], and by electrostatic entrapment during electropolymerization of polypyrrole [1, 4, 262].
For the electopolymerized PPy-ECH composites, the total crosslink density was 3.0 mol%, being 1.5 mol% by TEGDA and 1.5 mol% by MPB. The degree of hydration was measured using the thickness profilometer (n=5) both in PBS and DI water, Figure 3.9. In both cases, the initial degree of hydration of 68.6 (±0.8)% fell rapidly as PPy was added to the hydrogel. The addition of 1 mC/cm$^2$ of PPy resulted in a fall of the DoH from 66.4% to 24%. Further addition of PPy produced hydrogel composites with progressively decreasing degrees of hydration and which by 100 mC/cm$^2$ was essentially 0%. Since the degree of hydration is reflective of the free water content that occupies voids within the hydrogel [213], the loss of hydration suggests that the PPy is causing the expulsion of free water and may be growing within the voids of the hydrogel.
3.3.9 Modulus of PPy-ECH composites as a function of charge density

Nano-indentation measurements to reveal elastic modulus were taken from 500 randomly chosen points on each PPy-ECH modified transducer, Figure 3.10. Modulus values were determined for each charge density of electropolymerized polypyrrole and are summarized in Table 3.2. Freshly cast Py-hydrogel (0 mC/cm²) had a minimum of 1.5 mol% crosslink density via TEGDA with up to a maximum additional 1.5 mol% crosslink density via MPB following electropolymerization. Following electropolymerization of polypyrrole within the hydrogels the resulting PPy-ECH total crosslink density ranged from 2.5 - 4.5 wt% crosslinker. Hydrogels containing the electropolymerizable monomer had an elastic modulus of 56 (±32) kPa, which is in accord with that of a low crosslink density pHEMA hydrogel [213]. Modulus values for pHEMA at a pH of approximately 7.0 have been determined to range from 30-4000 kPa for crosslinker concentration ranging from 0.2 - 5.0 wt% [264]. Stiffness of the PPy-ECHs was shown to be tunable by controlling the charge density of electropolymerized polypyrrole. Monotonically increasing average elastic modulus was observed as electropolymerization charge density of polypyrrole was increased. Such increase in elastic modulus was similar in trend to that observed for increasing cross-link density [213]. Modulus values plateaued at 100 mC cm⁻² and averaged 499 (±293) kPa. Depending on the dopant used and charge density applied, the morphology and elastic modulus of bulk polypyrrole can vary greatly. Reported values of Young’s modulus for electrochemically formed, high charge density (>1 C cm⁻², ≈10 μm thickness) polypyrrole range from 10-1000 MPa [265, 266]. The low charge densities used in this
study generated PPy thickness equivalents on the nanometer scale (<250 mC cm\(^2\), <100 nm thickness). Therefore, we would not expect to observe an elastic modulus that was different than that of p(HEMA). We undertook some sensitivity analysis around the Poisson’s ratio, which for p(HEMA) is 0.29 and for PPy is 0.38 [266]. We find that for a Poisson’s ratio as large as 0.5 there is no significant difference in the measured modulus of the ECH.

**Figure 3.10.** The elastic modulus of PPy-ECH modified PME-Pt at varying charge densities of electropolymerized polypyrrole measured in PBS buffer (pH = 7.2) as determined by nano-indentation (n = 500 for each sample). *Insert:* Optical micrographs of the PPy-ECH on planar platinum electrodes.

**Table 3.2.** The elastic modulus of PPy-ECH composites as a function of varying electropolymerization charge density of polypyrrole used in the synthesis of the composite.

<table>
<thead>
<tr>
<th>Charge density of polypyrrole (mC cm(^2))</th>
<th>Average elastic modulus (kPa)</th>
<th>Standard Deviation (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>49</td>
<td>27</td>
</tr>
<tr>
<td>1</td>
<td>146</td>
<td>79</td>
</tr>
<tr>
<td>10</td>
<td>172</td>
<td>99</td>
</tr>
<tr>
<td>20</td>
<td>266</td>
<td>136</td>
</tr>
<tr>
<td>100</td>
<td>422</td>
<td>207</td>
</tr>
<tr>
<td>250</td>
<td>448</td>
<td>293</td>
</tr>
</tbody>
</table>
3.3.10 Electrical impedance spectroscopy of PPy-ECH composites as a function of charge density

The electrical impedance of the PPy-ECH composites was measured as a function of electropolymerization charge density over the range 0 - 900 mC/cm$^2$. Equivalent circuit modeling converged on a Randles-like network model that comprised a solution resistance, $R_s$, that represented the resistance of the PPy-ECH membrane (henceforth, $R_M$) in series with a constant phase element, CPE, that represented the capacitance of the co-planar arrangement of interdigitated electrodes, which was itself in parallel with a charge transfer, $R_{ct}$, that represented electron transfer events. Two different constructs were studied. The first was based on the presence of the seeding layer of PPy/PSSA that was directly formed on the platinum digits (bare Pt) of the IME device [Pt|PPy]. The seeding layer was typically fabricated by the application of 10 mC/cm$^2$ of electropolymerization charge density.

The resulting PPy/PSSA film served to provide a highly adherent layer on the metal from which surface initiated atom transfer polymerization to covalently attach the hydrogel layer proceeded. This layer was explored at different thicknesses. Figure 3.11(A) shows the trend in interfacial impedance magnitude and phase as increasing amounts of Py was electropolymerized. It is apparent that 10 mC/cm$^2$ was sufficient to dramatically change the interfacial impedance of the Pt microelectrodes of the IME device. Additional electropolymerization charge did not appreciably change the interfacial impedance until the application of 600 mC/cm$^2$. Finally, by 900 mC/cm$^2$ the film is established as a fully contiguous layer between the electrodes that produces a
purely Ohmic behavior and a frequency independence of the impedance magnitude. Thus 10 mC/cm\(^2\) was established as a suitable electropolymerization charge density to achieve a reduced interfacial impedance and subsequent hydrogel attachment.

Figure 3.11. Magnitude, \(|Z|\) (\(\Omega\)), and phase angle, \(\theta\) (degrees), of the electrical impedance as a function of electropolymerization charge density (0 - 900 mC/cm\(^2\)) for two different conductive polymer constructs on the electrodes of the IME 1025.3 M Pt. (A) The seeding layer of PPy on the platinum IME electrode [Pt|PPy] and (B) The growth of electropolymerized polypyrrole within a covalently attached hydrogel layer [Pt|PPy|PPy-ECH(EP)].

The second construct examined was electropolymerized PPy grown within the hydrogel layer covalently attached to the 10 mC/cm\(^2\) seeding layer [Pt|PPy|PPy-ECH]. Figure 3.11(B) shows the trend in interfacial impedance as increasing amounts of Py was electropolymerized within the hydrogel beginning from 0 mC/cm\(^2\) (i.e. Pt|Py-Gel) to 900 mC/cm\(^2\) (i.e. Pt|PPy-ECH). The presence of the hydrogel layer dramatically changed the
electrical dispersion characteristics and the impedance model. Further electropolymerization reduced the DC impedance confirming the addition of an electronically conductive component. The results of the equivalent circuit modeling produced the parameters summarized in Table 3.3 and Table 3.4.

Table 3.3. Equivalent circuit parameters for cleaned platinum IMEs as a function of electropolymerized polypyrrole charge density (Parentheses show ± 95% confidence interval of n = 3).

<table>
<thead>
<tr>
<th>Charge density (mC cm⁻²)</th>
<th>R_s (Ω)</th>
<th>error (%)</th>
<th>R_ct (kΩ)</th>
<th>error (%)</th>
<th>CPE-T (µF)</th>
<th>error (%)</th>
<th>CPE-P</th>
<th>error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Bare Pt)</td>
<td>69.3 (0.3)</td>
<td>2.09</td>
<td>4.8E+10 (1E12)</td>
<td>1.9E+07</td>
<td>0.13 (0.01)</td>
<td>1.85</td>
<td>0.94 (0.007)</td>
<td>0.27</td>
</tr>
<tr>
<td>10</td>
<td>102.9</td>
<td>2.30</td>
<td>9.9E+10</td>
<td>3.1E+08</td>
<td>4.94</td>
<td>2.59</td>
<td>0.81</td>
<td>0.60</td>
</tr>
<tr>
<td>50</td>
<td>85.0</td>
<td>1.00</td>
<td>1.5E+10</td>
<td>1.5E+08</td>
<td>16.89</td>
<td>1.45</td>
<td>0.80</td>
<td>0.38</td>
</tr>
<tr>
<td>100</td>
<td>80.5</td>
<td>0.65</td>
<td>9.7E+09</td>
<td>1.0E+08</td>
<td>23.16</td>
<td>0.94</td>
<td>0.78</td>
<td>0.26</td>
</tr>
<tr>
<td>150</td>
<td>78.8</td>
<td>0.69</td>
<td>4.9E+02</td>
<td>19.64</td>
<td>23.29</td>
<td>1.15</td>
<td>0.77</td>
<td>0.32</td>
</tr>
<tr>
<td>300</td>
<td>82.1</td>
<td>0.69</td>
<td>1.3E+01</td>
<td>2.20</td>
<td>45.66</td>
<td>1.69</td>
<td>0.80</td>
<td>0.46</td>
</tr>
<tr>
<td>600</td>
<td>77.2</td>
<td>0.58</td>
<td>4.6E-01</td>
<td>1.08</td>
<td>104.93</td>
<td>4.24</td>
<td>0.72</td>
<td>1.00</td>
</tr>
<tr>
<td>900</td>
<td>74.4</td>
<td>0.48</td>
<td>7.9E-02</td>
<td>1.24</td>
<td>178.77</td>
<td>9.57</td>
<td>0.67</td>
<td>2.06</td>
</tr>
</tbody>
</table>

Table 3.4. Equivalent circuit parameters for Py-Gel and PPy-ECH modified platinum IMEs as a function of electropolymerized polypyrrole charge density (Parentheses show ± 95% confidence interval of n = 2).

<table>
<thead>
<tr>
<th>Charge density (mC cm⁻²)</th>
<th>R_s (Ω)</th>
<th>error (%)</th>
<th>R_ct (kΩ)</th>
<th>error (%)</th>
<th>CPE-T (µF)</th>
<th>error (%)</th>
<th>CPE-P</th>
<th>error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Py-Gel)</td>
<td>79.4 (7.7)</td>
<td>1.45</td>
<td>1.4E+10 (3E+10)</td>
<td>6.5E+07</td>
<td>3.15 (0.10)</td>
<td>1.65</td>
<td>0.79 (0.019)</td>
<td>0.34</td>
</tr>
<tr>
<td>10</td>
<td>77.0 (4.9)</td>
<td>0.82</td>
<td>6.0E+10 (6E+09)</td>
<td>1.1E+08</td>
<td>7.26 (0.83)</td>
<td>1.07</td>
<td>0.83 (0.006)</td>
<td>0.24</td>
</tr>
<tr>
<td>50</td>
<td>73.2 (5.5)</td>
<td>1.07</td>
<td>7.0E+09 (1E+10)</td>
<td>5.2E+07</td>
<td>18.62 (0.58)</td>
<td>1.78</td>
<td>0.83 (0.013)</td>
<td>0.45</td>
</tr>
<tr>
<td>100</td>
<td>72.5 (4.8)</td>
<td>0.53</td>
<td>2.3E+01 (9E+00)</td>
<td>1.49</td>
<td>24.28 (1.21)</td>
<td>1.22</td>
<td>0.82 (0.003)</td>
<td>0.30</td>
</tr>
<tr>
<td>150</td>
<td>72.0 (6.3)</td>
<td>0.51</td>
<td>4.6E+00 (7E-01)</td>
<td>0.86</td>
<td>22.71 (7.00)</td>
<td>1.79</td>
<td>0.82 (0.001)</td>
<td>0.38</td>
</tr>
<tr>
<td>300</td>
<td>71.5 (6.9)</td>
<td>0.49</td>
<td>4.6E-01 (3E-01)</td>
<td>0.81</td>
<td>52.74 (6.86)</td>
<td>3.92</td>
<td>0.81 (0.009)</td>
<td>0.79</td>
</tr>
<tr>
<td>600</td>
<td>69.6 (6.8)</td>
<td>0.51</td>
<td>6.2E-02 (2E-02)</td>
<td>1.43</td>
<td>111.51 (22.29)</td>
<td>12.79</td>
<td>0.77 (0.027)</td>
<td>2.40</td>
</tr>
<tr>
<td>900</td>
<td>68.5 (6.2)</td>
<td>0.57</td>
<td>1.8E-02 (9E-04)</td>
<td>3.58</td>
<td>153.13 (22.83)</td>
<td>39.37</td>
<td>0.77 (0.021)</td>
<td>6.65</td>
</tr>
</tbody>
</table>
Bare platinum transducers had a capacitance of 0.13 (±0.01) µF. Applying even a low charge density of PPy (10 mC/cm²) directly onto the platinum electrodes increased the capacitance parameter, CPE-T, by over an order of magnitude, Figure 3.12(A). Modification with PPy-ECH resulted in significantly higher capacitance for all charge densities when compared to bare platinum (p < 0.003). Capacitance is known to increase in proportion to polypyrrole film thickness [267]. The difference in capacitance between [Pt|PPy] and [Pt|PPy|PPy-ECH] became negligible at charge densities ≥100 mC/cm². The porous structure of polypyrrole provides a high surface area and behaves as an electrochemical double layer capacitor with counter ions screening charge [268, 269].

The constant phase element deviated from an ideal capacitor was determined by the decreasing CPE-P parameter after application of a polypyrrole seeding layer and modification with PPy-ECH at all charge densities. The CPE-P value being less than 1 (ideal capacitor) for the bare platinum IME is likely due to the co-planar geometry of the transducer electrodes vs. the traditional parallel plate arrangement.

Figure 3.12. A) Capacitance (CPE-T) and B) Membrane resistance (R_M) as a function of electropolymerized polypyrrole charge density applied to the bare platinum and PPy-ECH modified interdigitated microsensor electrodes (IME 1025.3 M Pt).
The membrane resistance parameter, $R_M$, of the electrochemical interface is a likely mix of ionic and polaronic conductivity and thus a function of electrolyte concentration, temperature, intrinsic resistance of the active material, the dimensions of the electrodes and cell, and the contact resistance between materials [270]. The true $R_S$ defined as the bulk solution resistance, was determined from use of the bare Pt IME and found to be 69.3 ($\pm$0.3) $\Omega$. Since temperature (25 °C) and electrolyte concentration were kept constant, any variation in $R_M$ ($\Omega$) may be attributed to changes in electrode dimensions and/or changes in the conductivity of the membrane materials. As seen in Figure 3.12(B), the presence of the Py-Gel membrane increases the resistance relative to that of the solution resistance ($R_M$(Py-Gel) = 80 $\Omega$ and $R_S$ = 69.3 ($\pm$0.3) $\Omega$). This was expected as the ionic conductivity of the solution is higher than that of the hydrogel. However, following initiation of electropolymerization, the resistance of the Pt|PPy construct becomes immediately higher than that of the Pt|PPy|PPy-ECH(EP) construct. For low charge densities (<100 mC/cm$^2$) we observed higher membrane resistances for Pt|PPy that extend beyond the 95% confidence intervals of Pt|PPy|PPy-ECH(EP). These results indicate higher conductivity for PPy-ECH compared to PPy. The apparent membrane resistance for both constructs approached the original bulk solution resistance as the electropolymerization charge density was increased. This evidence suggests the presence of high conductivity pathways being formed within the ECH.

The impedance magnitude in the low charge density domain was shown to be primarily a function of the double layer capacitance and solution resistance. No charge transfer was observed on bare, low charge density seeded, Py-Gel and/or PPy-ECH
transducers indicating an absence of redox events to support charge injection into and out of the membrane. The % error for charge transfer resistance indicated a good fit at charge densities ≥300 mC/cm$^2$ and ≥100 mC/cm$^2$ for the Pt|PPy and Pt|PPy|PPy-ECH(EP), respectively. The change in R$_{ct}$ indicates a change in impedance response from being purely capacitive double layer charging to direct charge transfer across the Pt|PPy or PPy|Electrolyte interface. Visual inspection of the IME surfaces confirms the efficiency in bridging the fingers of the platinum IME device with polypyrrole for PPy-ECH modified transducers compared to PPy seeded platinum transducers, **Figure 3.13**, as was previously reported [224]. These results indicate that the transition from ionic to polaronic conduction occurs earlier in the PPy-ECH construct and that hydrogel facilitates long-range electrical conductivity and interconnectivity of the formed interpenetrating network of polypyrrole.

![Figure 3.13](image)

**Figure 3.13.** Optical micrograph showing the bridging of polypyrrole, or lack thereof, between the electrode digits as a result of increasing electropolymerized polypyrrole charge density for IMEs with (A) polypyrrole seeding and (B) PPy-ECH modification.
The rapid decrease in charge transfer resistance of PPy-ECHs may be due to the dimensionality of the polypyrrole structure formed within the void spaces of the hydrogel during electropolymerization. The decreased hydration and void volume of the PPy-ECH as charge density increases supports this observation. Conduction via Mott’s variable range hopping (VRH) has been well established as the dominant mechanism of polaronic charge transport for polypyrrole, and the conduction mechanism in pristine polypyrrole is three dimensional VRH where electrons hop in a probabilistic manner between localized polaron states in all directions [175, 176, 271-273]. Forming composite materials with polypyrrole have been shown to lead to one dimensional VRH with faster intra-chain charge hopping [273]. Highly ordered CEP chains with few inter-chain links will have less instances of inter-chain charge hopping and more intra-chain charge hopping leading to greater conductivity. Future studies focused on the temperature dependence of conductivity for the electroconductive hydrogels may be performed to evaluate the mechanism of conduction and dimensionality of electron hopping [272].

3.3.11 Anchorage-dependent cell growth on Py-hydrogels and PPy-ECH hydrogel composites

In previous work we have reported the considerable growth and proliferation of anchorage-dependent human muscle fibroblasts (RMS 13) and murine pheochromocytoma cells (PC12) on electroconductive blends of poly(HEMA-co-PEGMA-co-HMMA-co-SPMA) and poly(Py-co-PyBA) [102]. In that work, PC12 and RMS13 cells were seeded onto Au*|Gel, Au*|PPy, and Au*|PPy-ECH surfaces with electropolymerization times on the order of 5, 25 and 50 s corresponding to 11, 87 and
250 mC/cm² (an asterisk identifies a functionalized gold surface). The PC12 cell line showed strong positive correlation between proliferation and the extent of electropolymerization. A concern has been whether or not the observed growth and proliferation arose from the evolving modulus of the PPy-ECH membrane or of the evolving electrical properties.

Figure 3.14. A) The percent change in cell proliferation of RMS 13 and PC12 cells following four-day incubation on Au*|hydrogel, Au*|PPy, and Au*|PPy-ECH plotted alongside the elastic modulus of the three surfaces. B) The percent change in cell proliferation of PC12 cells following four-day incubation on Au*|hydrogel, Au*|PPy, and Au*|PPy-ECH of different electropolymerization charge densities [5s (11mC/cm²), 25s (87mC/cm²) and 50s (250 mC/cm²)] plotted alongside the elastic modulus of the various surfaces. Cell seeding density was 4.9+/−0.7 x10⁵ cells/ml.

Figure 3.14(A) shows the percentage change in both RMS13 and PC12 cell numbers after four days of culture on the various surfaces plotted alongside the elastic modulus. Both RMS13 and PC12 cell lines revealed a decrease in cell numbers on the Au*|Gel and Au*|PPy surfaces, while both cell types showed increases on the Au*|PPy-ECH (50 s or 250mC/cm²). For RMS13, there was a 50 to 70% decrease in cell density after 4 days on the Au*|Gel and Au*|PPy surfaces respectively, while there was a nearly 80% increase in cell density on the Au*|PPy-ECH (50 s or 250mC/cm²). For the PC12, there was a 70 to 90% decrease in cell density after 4 days on the Au*|Gel and Au*|PPy surfaces, while there was a 12% increase in cell density on the Au*|PPy-ECH (50 s or
250mC/cm²). PC12 cell proliferation on Au*|PPy-ECH was nearly 70% less than RMS13. The reason for this difference is unclear, but it may be related to differences in how these two cell lines attach to the substrate surface and/or differences in their rates of cell division. A two-tailed student’s t-test indicated a statistically significant change in both RMS13 and PC12 cell populations from Day 0 to Day 4 (p<0.05) for all samples with the specific exception of PC12 on Au*|PPy-ECH (50s) (corresponding to 250 mC/cm²), which had a p-value of 0.26. This indicates that there was little to no proliferation of PC12 during the 4 day incubation period on this particular surface. From Figure 3.14(A), one also sees that the elastic modulus of the Gel-PPy film is nearly an order of magnitude greater than either the native hydrogel (Gel) or polypyrrole (PPy). The positive correlation between cell proliferation and elastic modulus of the film exemplified herein suggests that stiffness of the substrate plays an important role in cell attachment and subsequent cell division. A critical threshold in the elastic modulus is likely necessary to be surpassed in order for positive cell proliferation to occur, and may also be dependent on cell type. This critical threshold is yet to be determined; however a suggested threshold value is reported later in this text.

As in Figure 3.14(A), Figure 3.14(B) also shows the percentage change in PC12 cell numbers measured following trypsinization after four days of culture on the various surfaces plotted alongside the elastic modulus. In this instance, however, proliferation was investigated as a function of Gel-PPy electropolymerization times - 5, 25 and 50 s, corresponding to 11, 87 and 250 mC/cm² to further validate the hypothesis that proliferation is directly correlated to substrate stiffness. For the native hydrogel (Gel) and
PPy films, there was a 70-80% decrease in the number of PC12 cells four days following the initial cell culture, which is consistent with observations made in the previous experiment, Figure 3.14(A). On the Gel-PPy films (ECH films), however, an increase in cell density of about 5% was observed for Au*|PPy-ECH (50s) and 40-60% for Au*|PPy-ECH (5 and 25s). A two-tailed student’s t test indicated a statistically significant change in the PC12 cell population from Day 0 to Day 4 (p<0.05) for all samples with the exception of Au*|PPy-ECH (50s) (corresponding to 250 mC/cm²) which had a p value of 0.737. This is also consistent with the results observed for PC12 in the previous experiment, Figure 3.14(A). The increasing elastic modulus of the electroconductive hydrogel with electropolymerization charge density is a direct indication of the increase in % PPy content of the ECH (also seen in the UV absorbance spectrum, Figure 3.8). However, the cell proliferation studies appear to indicate that there are fundamental differences in the material properties experienced (seen) by the cells as they attempt to attach to the surfaces of the hydrogel, PPy and PPy-ECH respectively and that these differences are not captured solely by the elastic modulus. Although the ECH has been described as a hybrid material combining the poly(HEMA)-based hydrogels with the conducting polymer polypyrrole, it appears that the surface of the ECH may have a different molecular make-up from either the native poly(HEMA)-based hydrogel or the polypyrrole. In the case of both the Au*|PPy-ECH (5s) and Au*|PPy-ECH (25s), there was a statistically significant net increase in cells, while there was a net decrease for Au*|Gel and Au*|PPy. If the PPy-ECH samples were truly homogeneous, isotropic hybrids of both the hydrogel and PPy, one would expect that in all these cases, there
would be a net decrease in cell density, since these component materials have been shown to resist protein adsorption and consequently inhibit cell attachment and proliferation. However, there is a clear increase in the Au*|PPy-ECH (5s) and Au*|PPy-ECH (25s) samples which might be associated with the increased elastic modulus (stiffness) of the composite conducting hydrogel films. As the % PPy content of the ECH increases beyond a certain threshold, the stiffness may play less of a role and its attributes for cell anchorage diminished compared to the material properties afforded by the PPy. This elastic modulus threshold necessary for PC12 cell proliferation should at least fall between 150 and 300 kPa, according to Figure 3.14(B). The fact that there was no appreciable cell proliferation on the final sample, Au*|PPy-ECH (50s) likely indicates that the surface properties of the material, at this point, are more similar to PPy than to the ECH composite material and that the PPy dominated molecular make up of this material likely overcomes the ability of the elastic modulus to influence cells to proliferate. It is unlikely that surface topography (roughness) is at play, since profilometry results (not shown) reveal little variability across ECH films of different electropolymerization charge densities.
Figure 3.15. Schematic illustration of the possible role of the elastic modulus of the PPy-ECH on the growth and proliferation of attachment dependent cells.

Figure 3.15 provides a schematic illustration of how we conceptualize the competing influences of the cellular response to the elastic modulus or stiffness of the substrate with the competing response of the PPy-ECH growth. During the early stages of electropolymerization (1-50 mC/cm$^2$) the PPy is electropolymerized within void spaces, normally occupied by water, within the hydrogel. Hence, the hydration properties of the hydrogel are rapidly compromised as bulk water is expelled. During the middle stages of electropolymerization (50-100 mC/cm$^2$) the PPy interpenetrates the hydrogel network. During the late stages of electropolymerization (100-250 mC/cm$^2$) the PPy begins to grow on the surface of the hydrogel layer and reestablishes its cell-surface interaction but displaces a modulus reminiscent of the composite beneath.

**3.3.12 Effects of a polypyrrole seeding layer on analytical performance of biotransducers**

To create effective, stable and reproducible biotransducers, it is necessary to establish an intimate and stable interface between the biorecognition membrane layer and
the underlying physicochemical transducer, in this case, the electrode. To achieve this we developed an approach based on the fabrication of a nano-dimensional seeding layer of pristine polypyrrole-polystyrene sulfonate (PPy(PSSA)) on to which a biosmart hydrogel can be covalently coupled. The effects of seeding transducers with PPy(PSSA) prior to subsequent electropolymerization and entrapment of enzymes was explored. A low charge density of 10 mC/cm² was used as a seeding layer prior to applying 100 mC/cm² of PPy/GOx. The enzyme kinetic parameters of biotransducers were determined using Lineweaver-Burke analysis. Unseeded biotransducers had a sensitivity of 0.77 (±0.35) µA cm⁻² mM⁻¹, an I_max of 16.8 (±6.5) µA cm⁻², a linear dynamic range maximum of 15.8 (±2.8) mM, a K_Mapp of 11.1(±4.3) mM, and a limit of detection of 0.07 (±0.05) mM. Seeded biotransducers had a sensitivity of 1.44 (±0.78) µA cm⁻² mM⁻¹, an I_max of 35.3 (±18.3) µA cm⁻², a linear dynamic range maximum of 19.4 (±1.0) mM, a K_Mapp of 11.8 (±0.3) mM, and a limit of detection of 0.04 (±0.03) mM.

**Figure 3.16.** The effects of the PPy(PSSA) seeding layer (10 mC/cm²) on the enzyme kinetic parameters of biotransducers fabricated at 100 mC/cm² (error bars show 95% confidence intervals, n ≥ 5). * = P < 0.05; ** = P < 0.01.
Figure 3.16 shows the effects of the PPy(PSSA) seeding layer (10 mC/cm²) on the enzyme kinetic parameters of biotransducers fabricated at 100 mC/cm². Both the sensitivity and the apparent Michaelis-Menten enzyme kinetic parameters were statistically equivalent suggesting no deleterious influence of the adhesion promoting seeding layer on biotechnical performance. The linear range of an amperometric biosensor system is typically a function of analyte diffusivity through the bioactive membrane [274]. The linear dynamic range of seeded biotransducers was not significantly broader than for the unseeded. The nano-thin PPy(PSSA) layer was not enough to serve as a barrier to diffusion for hydrogen-peroxide in order to increase the linear range or adversely affect other enzyme kinetic parameters. The $K_{\text{Mapp}}$ values determined show that no changes to enzyme affinity occurred as a result of the seeding layer. Seeded biotransducers showed less variability in $K_{\text{Mapp}}$ indicating good reproducibility of enzyme affinity compared to unseeded transducers. For amperometric, enzyme-based biosensors, the maximum current is related to total enzyme activity [1]. The nearly 2-fold increase in maximum current was statistically significant ($p = 0.045$). These results are a good indication that the seeding layer serves to better preserve enzyme active site structure and function but does not lead to an increase in the number of enzymes entrapped as sensitivity does not significantly increase. It is likely that the seeding layer acts as a soft milieu onto which enzymes may then be adsorbed and entrapped within a PPy|PPy interface after subsequent electropolymerization. Polypyrrole is known to have a stabilizing influence on enzymes [223]. Enzymes being immobilized
onto an unseeded electrode will be entrapped within a mechanically disparate interface of PPy|Pt that may not preserve the structure of immobilized enzymes as effectively.

The principal role of the seeding layer was to be an interface for hydrogel casting. However, the seeding layer was shown to significantly decrease the average biofabrication time from 68 (±22) min for the unseeded biotransducer to 35 (±6) min for the seeded biotransducer (p = 0.007); a reduction of 50%. Electropolymerization efficiency is an important biofabrication parameter. Enzymes require a hydrated, protective environment in order to function in vivo [4]. Hydrogels provide the necessary protective hosting for biomolecules [275] and the in vivo biocompatibility for the protection of the biotransducers [4]. However, hydrogel modified transducers require considerable overpotential for electropolymerization; as much as 1,000 mV vs. Ag/AgCl and the polymerization times are comparable to that of unmodified electrodes [12].

3.3.13 Enzyme monomerization and sulfonization

Lysine groups of glucose oxidase were covalently coupled to either pyrrole butyric acid (PyBA) or sulfobenzoic acid (SBA) using EDC-NHS coupling chemistry, Figure 3.17. The amperometric dose response of biotransducers to glucose was measured for i) covalently conjugated systems: PPy(SBA-con-GOx), P(Py-co-PyBA-con-Gox), ii) non-covalently conjugated systems: PPy(SBA)-GOx, P(Py-co-PyBA)-GOx, and iii) control systems: PPy-GOx. The covalently conjugated systems with a 10:1 molecular ratio of PyBA:GOx or SBA:GOx are depicted in Figure 3.18. The approach to covalent coupling considered in this work is intended to be a simple and effective means of enzyme conjugation that can be performed in a single solution and immediately utilized
for electropolymerization without need for separations. Monomerized enzymes were prepared with the intent of having them be directly incorporated into the backbone of the forming electropolymerized polypyrrole. Sulfonized enzymes were prepared in order to have them act as more effective dopants and be electrostatically entrapped within the positively charged polypyrrole backbone. Biosensor performance parameters for all systems are summarized in Table 3.5.

**Figure 3.17.** Scheme of enzyme modification using EDC-NHS coupling chemistry to monomerize glucose oxidase with PyBA; Sulfonization of enzymes with SBA was performed in a similar fashion.
Figure 3.18. Subunit of glucose oxidase from Aspergillus niger with Lysine groups in red (top), monomerized enzymes used for preparing P(Py-co-PyBA-con-Gox) (bottom left) and sulfonized (bottom right) enzymes used for preparing PPy(SBA-con-Gox) with 10:1 ratio of PyBA or SBA conjugation to enzymes.

3.3.14.1 Effects of enzyme modification on kinetics of electropolymerization

The kinetics of electropolymerization (time to reach 100 mC/cm\(^2\) of charge) were compared for all systems. The control systems of PPy-GOx had an electropolymerization time of 68 ±22 min. Enzyme systems P(Py-co-PyBA)-GOx and P(Py-co-PyBA-con-Gox) both had significantly greater (p<0.03) electropolymerization times compared to PPy-GOx. Enzyme systems PPy(SBA)-GOx, PPy(SBA-con-Gox) had significantly lower electropolymerization times compared to all other systems (p<0.02). Sulfobenzoic acid is a highly mobile dopant capable of screening charge and making electropolymerization kinetics more facile for polypyrrole [276]. No significant difference in kinetics was noted between covalent and non-covalent conjugation for either PyBA or SBA systems. A decrease in variability in electropolymerization time was observed for the covalently conjugated systems when compared to the non-covalently conjugated systems. There is
likely competition in doping of the formed polypyrrole between adsorbed glucose oxidase and PyBA/SBA in the non-covalent systems, whereas for covalently conjugated systems the dopant becomes solely the adsorbed monomerized or sulfonized enzyme.

Table 3.5. Dose response (PBS, pH = 7.2, 25 °C) performance parameters for biotransducers fabricated with covalently modified enzymes, non-covalently modified enzymes and control systems having no modification (± standard deviation is shown).

<table>
<thead>
<tr>
<th>[E] = 1.0 mg/mL</th>
<th>PPy-GOx (n = 4)</th>
<th>PPy(SBA)-GOx (n = 4)</th>
<th>PPy(SBA-con-GOx) (n = 2)</th>
<th>P(Py-co-PyBA-GOx) (n = 2)</th>
<th>P(Py-co-PyBA-con-Gox) (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerization time to 100 mC/cm² (min)</td>
<td>68 ±22</td>
<td>23 ±19</td>
<td>15 ±5</td>
<td>204 ±37</td>
<td>230 ±132</td>
</tr>
<tr>
<td>Sensitivity (µA/mM/cm²)</td>
<td>0.87 ±0.04</td>
<td>1.8 ±1.2</td>
<td>1.9 ±0.2</td>
<td>4.8 ±0.9</td>
<td>0.99 ±0.28</td>
</tr>
<tr>
<td>Response time (s)</td>
<td>41 ±21</td>
<td>17 ±9</td>
<td>18 ±5</td>
<td>17 ±5</td>
<td>14 ±5</td>
</tr>
<tr>
<td>Detection limit (mM)</td>
<td>0.50 ±0.30</td>
<td>0.01 ±0.01</td>
<td>2.2E-3 ±1.8E-3</td>
<td>0.05 ±0.03</td>
<td>0.02 ±0.01</td>
</tr>
<tr>
<td>K_Mapp (mM)</td>
<td>14.1 ±3.1</td>
<td>8.2 ±2.6</td>
<td>11.1 ±5.6</td>
<td>7.2 ±2.6</td>
<td>19.7 ±3.9</td>
</tr>
<tr>
<td>I_max (µA/cm²)</td>
<td>23.3 ±3.1</td>
<td>30.6 ±18.8</td>
<td>39.2 ±11.7</td>
<td>77 ±25</td>
<td>28.9 ±4.0</td>
</tr>
<tr>
<td>Linear range (mM)</td>
<td>17.4 ±7.0</td>
<td>9.4 ±2.6</td>
<td>10.2 ±3.2</td>
<td>9.5 ±2.8</td>
<td>25.6 ±5.6</td>
</tr>
</tbody>
</table>

3.3.14.2 Biotransducer performance with sulfonized enzymes

The covalently sulfonized system, PPy(SBA-con-GOx), had a sensitivity of 1.92 ±0.2 µA/mM/cm², a response time of 18 ±5 s, a detection limit of 2.2E-3 ±1.8E-3 mM, a K_Mapp of 11.1 ±5.6, a maximum current of 39.2 ±11.7 µA/cm² and a linear range of 10.2 ±3.2 mM. The PPy(SBA-con-GOx) system was significantly more sensitive (p<0.0002) than the PPy-GOx system. It also had the best limits of detection of all the systems with significantly better (p=0.002) resolution compared to P(Py-co-PyBA-con-Gox). Response times were found to be similar for all systems. The non-covalently coupled
SBA system, PPy(SBA)-GOx, had a sensitivity of $1.8 \pm 1.2 \ \mu A/mM/cm^2$ which was not found to be different from PPy-GOx controls. Electropolymerized polypyrrole glucose biotransducers prepared with GOx conjugated to poly(2-acrylamido-2-methylpropane sulfonic acid) polyanion dopants have been shown to have sensitivity ranging from 180-270 nA/mM/cm$^2$ with a linear range of 20-26 mM and a Michaelis-Menten constant of 46.7 mM [277, 278]. Overall the covalent sulfonization process successfully yielded biotransducers with higher sensitivity but at the cost of decreased linear dynamic range when compared to the GOx-polyanion system found in literature.

### 3.3.14.3 Biotransducer performance with monomerized enzymes

The non-covalently P(Py-co-PyBA)-GOx system had a sensitivity of $4.8 \pm 0.9 \ \mu A/mM/cm^2$, a response time of $17 \pm 5$ s, a detection limit of $0.05 \pm 0.03$ mM, a $K_{\text{Mapp}}$ of $7.2 \pm 2.6$, a maximum current of $77 \pm 25 \ \mu A/cm^2$ and a linear range of $9.5 \pm 2.8$ mM. Its sensitivity was significantly greater than every biotransducer (p<0.045) other than PPy(SBA-con-GOx). P(Py-co-PyBA)-GOx had the highest maximum current of all systems but was only significantly greater (p<0.01) than PPy-GOx systems. Covalently monomerized GOx did not perform statistically different from PPy-GOx systems. Glucose oxidase that is lysyl modified with an N-substituted 1H-Pyrrole-1-propionic acid have been shown to have higher electrode activity compared to native GOx in polypyrrole membranes [279]. Chemical modification of GOx lysine groups with 3-carboxymethyl pyrrole has also been shown to exhibit higher enzyme activity and storage stability than systems using N-substituted pyrrole [280]. Our results show that
incorporation of PyBA without covalent coupling is sufficient for increasing biosensor performance with respect to higher sensitivity and overall enzyme activity.

**3.4 Conclusions**

It is highly feasible to fabricate amperometric enzyme biotransducers using the procedure of Py electropolymerization to ensnare GOx and immobilize it within hydrogel membrane layers that were covalently anchored onto surface modified platinum electrodes. The electropolymerization charge density dependence of ECH-modified biotransducer sensitivity and enzyme activity revealed a maximum sensitivity of 281 nA/mM/cm$^2$ and an apparent $K_M$ of 33 mM at an applied charge density of 1.0 mC/cm$^2$. Screening of ascorbic acid was consistent with previously reported results. Following electropolymerization at low charge density the partitioned and adsorbed enzymes within the hydrogel may become trapped, have more enzyme active sites available for reaction, less diffusional constraints, but will be more unstable. Electrodes of 10 mC/cm$^2$ gels were capable of entrapping enzymes within the hydrogel and polypyrrole, maintain their affinity, but also lead to an increase in diffusional barriers for enzyme substrate and products. A tradeoff between enzyme stability over time and magnitude of current response over time appears to be necessary. It is concluded that enzyme incorporation into hydrogels via electropolymerization of polypyrrole is an effective means of imparting bioactivity to a transducer, but the biotransducer becomes more sensitive to the mass transport limitations imparted by the overoxidized polypyrrole. This study optimized biotransducer performance as a function of electropolymerized polypyrrole. There is need to investigate and optimize the relationship between the crosslink-density
and the electopolymerization charge density in order to achieve a strategic balance between the compromise in sensitivity and the benefits of interference screening, biocompatibility and long term enzyme stability. When modified with over-oxidized polypyrrole, the transducer was shown to reject access of the negatively charged FeCOOH, analogous to interference shielding of ascorbic acid. A temporal improvement in biotransducer sensitivity, regardless of charge density of fabrication, was associated with the presence of the hydrogel membrane layer. Such increases in sensitivity indicate increases in immobilized enzyme activity over time.

An interpenetrating co-network of a pHEMA-based hydrogel possessing a variety of modifying monomers and conductive, electroactive PPy-co-PyPA was synthesized by covalently linking the two networks via a hetero-bifunctional monomer, 2-methacryloyloxyethyl-4(3-pyrrolyl)butanate (MPB). MPB was synthesized in situ from the conjugation of PyBA (acid) and AEMA (amine). The elastic moduli of the electroconductive interpenetrating co-network were larger than either the Py-Gel or PPy only and the optical density increased linearly while the stiffness increased and plateaued with electropolymerization charge density. The frequency dependence of the electrical impedance of the PPy-ECH co-network showed a dramatically different dispersion compared to the Py-Gel and to PPy. Increasing charge density did not appreciably change this dispersion. Attachment dependent RMS13 and PC12 cells demonstrate a strong correlation of their growth and proliferation with the elastic modulus of the electroconductive hydrogels. No such correlation was observed with respect to either the membrane resistance, membrane capacitance or charge transfer resistance of the film.
However, the evolving molecular composition and structure of the surface may also play an important role in supporting cell growth and proliferation. The interplay among the elastic modulus and the PPy content may be at work in determining the ability of cells to proliferate on the PPy-ECH materials.

Seeding platinum electrodes with polypyrrole had no significant effect on sensitivity, linear range, limits of detection and response time while increasing maximum current. These results indicate that entrapping enzymes onto polypyrrole seeded electrodes better maintains activity compared to entrapment directly onto electrodes. Seeded transducers also had significantly more facile polypyrrole electropolymerization kinetics. The seeding layer may be a good option for making the polymerization kinetics more facile with a lesser overpotential in these hydrogel systems when highly mobile dopants (Cl, HPO\textsubscript{4}\textsuperscript{2-}) are not available. Sulfonization of enzymes was shown to be a fast and effective means of improving biotransducer performance. Sulfonization using sulfobenzoic acid at a 10:1 SBA:GOx ratio was shown to be useful in making electropolymerization kinetics more facile and doubling biotransducer sensitivity compared to native enzyme systems. Monomerization of enzymes using pyrrole butyric acid at a 10:1 PyBA:GOx ratio did not significantly influence biotransducer performance. Non-covalently incorporated pyrrole butyric acid dramatically increased biotransducer sensitivity and decreased linear dynamic range.
4.1 Introduction

4.1.1 Lethality of trauma and the need for advanced trauma diagnostics

Hemorrhage alone accounts for nearly 50% of deaths within the first 24 hours of trauma care, Fig. 4.1 [281, 282]. Hemorrhage is also the #1 preventable cause of death for warfighters [13] and accounts for 68% of all battlefield fatalities. Complications such as systemic inflammatory response syndrome, compensatory anti-inflammatory response syndrome, multiple organ failure, and multiple organ dysfunction syndrome will arise in the re-perfused hemorrhage patient [283]. These complications may further result in permanent organ damage and possibly death. Caregivers must be judicious in their resuscitation in order to save the hemorrhaged trauma patient while simultaneously avoiding these complications [33, 284]. This becomes especially difficult under austere conditions (highway or battlefield) where first responders have no technology for diagnosing and monitoring patients (for occult (undetectable) hemorrhagic shock) with hypotension [21]. A gap exists in diagnostic technology for first responders and caregivers when compared to the capabilities of a trauma center or emergency room [124]. Hemorrhage produces a state of insufficient oxygen delivery or consumption and decreased cellular perfusion that leads cellular metabolism into anaerobic respiration [285]. The extent of hypoperfusion depends on the rate of blood loss, total amount of blood loss, duration of tissue vasoconstriction, the level of perfusion pressure in the blood
vessels, and the type of vascular bed being affected [25]. Resuscitation with whole blood, crystalloids or colloids, releases the products of tissue ischemia into systemic circulation where they affect other host responses and tissues [32, 283]. There is thus pressing need to develop technology solutions to address the first 24 hours following trauma-associated hemorrhage in order avoid such outcomes as hemorrhagic shock.

![Bar chart comparing time and mechanisms of traumatic death](image)

**Figure 4.1**. Bar chart comparing time and mechanisms of traumatic death. Nearly all deaths due to hemorrhage occur within the first 24 hours. Major clinical complications of hemorrhage and hemorrhagic shock such as sepsis, organ failure and multiple organ dysfunction syndrome replace hemorrhage as a major cause of death [281].

### 4.1.2 Biosensors as point of care technology for continuous patient monitoring

The use of point-of-care technology to diagnose and stratify trauma victims beginning at the point of injury is gaining attention and acceptance [3, 117, 286]. Unlike traditional point of care testing devices, like hand held monitors with test strips and/or cartridges, fully integrated *in-vivo* biosensors are special point-of-care testing platforms upon which biological events may be observed and measured in a continual manner. The implantable biosensors of the current proposal use means of sensing similar to that performed at the lab bench, but the (testing) data acquisition is faster, continual, uses a smaller footprint instrument, is enabled with telemetry capabilities, and is done within the
A rapidly deployable biosensor system administered by a first responder can begin immediate and continual monitoring of metabolic vital signs, logging data during transport and handing this off to a medivac vehicle, then to the hospital trauma center in order to enable monitoring right through to the Intensive Care Unit (ICU).

4.1.3 New patient data trends available to care givers

Rapidly and continually measuring metabolic biomarkers such as glucose and lactate will permit the consideration of new trends in patient data analysis. Two major novel data trends include “rate of change in analyte concentration” and “integral exposure dose above normal concentrations of analytes”. These data trends are not readily observed using infrequent laboratory blood (stat) analysis in a hospital environment where turnaround times may be 30-60 minutes [117, 119, 122]. Such data trends on glucose and lactate simultaneously have yet to be rigorously and prospectively explored in the musculature during trauma. Though hand-held blood analyzers have the capability of measuring glucose and lactate at ca. 2-4 minutes per test [122], there is significant cost for each test (ca. $15) with tests requiring periodic blood sampling of patients, which is less than ideal for patients who have suffered blood loss [287]. A well designed implantable biosensor system will be more efficient at capturing data over long periods of time compared to traditional blood analysis. These new data analysis trends may be used to develop new resuscitation initiation criteria and regimens for caregivers to utilize.

During trauma and hemorrhage, blood within the vasculature is in a well-studied compartment of the body. Less attention has been given to other compartments due to
their lack of access and the invasiveness required in reaching them. A minimally invasive biosensor, however, can readily be implanted into skeletal muscle using a large gauge needle. A biosensor, implanted by the first responder at the point of injury, will enable immediate and continuous measurement of a trauma patient’s physiological status. Decisions on care are made based on physical and metabolic vital signs that a patient presents, knowledge gathered by emergency first responders, and the experience of medical staff. Data and trends in patient status gathered wirelessly can automatically be handed off to and integrated with a hospital’s WiFi network.

4.1.4 Use of the MDEA 5037 biosensor system for monitoring the events of trauma

The dual responsive Electrochemical Cell-on-a-Chip Microdisc Electrode Arrays (ECC MDEA 5037) is a recently developed transducer for use in a wireless, implantable biosensor system for the continuous measurement of interstitial analytes. Preliminary studies with the MDEA 5037 in a rat hemorrhagic shock model have shown discordance between blood and interstitial lactate levels, Figure 4.2 [3]. Lactate can accumulate more readily in the muscles especially during periods of compensation and increased peripheral resistance during moderate to severe hemorrhage as blood oxygen delivery will be even further reduced, thus causing a rapid spike in interstitial lactate levels. Given knowledge of the Cori cycle, this lactate will diffuse back into the blood and eventually make its way to the liver [130, 288]. It is hypothesized that under conditions of diminished peripheral perfusion, lactate levels in the tissues will be discordant with systemic lactate levels, and that the amount and duration of the tissue lactate levels will be a better indicator of the
extent of hemorrhagic shock in the trauma patient. Continued examination of interstitial compartments using biosensors will aid in understanding the temporal relationships among markers of stress in these environments and how they relate to shock-like states.

**Figure 4.2.** A) The ECC MDEA 5037 is a dual channel biotransducer that is interfaced with a wireless potentiostat. B) In preliminary studies, intramuscularly implanted lactate sensitive MDEA 5037s have shown the difference between interstitial and blood lactate levels.

The ultimate goal of this research is to change patient survival outcomes for victims of trauma associated hemorrhage. Trauma is a leading cause of death among our youth, both on our highways and on our battlefields. The aim is to deploy an *in vivo* (intramuscular) biosensor system (The PSM Biochip) that will allow immediate and continual pre-hospital monitoring of lactate and glucose to inform patient specific interventions that will improve survivability from hemorrhage (and hemorrhagic shock), Fig. 4.3. The following work is a preliminary study of wireless biosensor systems *in vivo* to evaluate initial functionality.
Figure 4.3. An implantable biosensor for continual monitoring will improve trauma outcomes by engaging pre-hospital monitoring, providing adjunct metabolic vital signs to first responders, and enabling more physiologically based care and patient specific interventions. Such a system can be rapidly deployed by emergency medical services (EMS) during mass inpatient care (MIC) from natural disasters and in the theater of war by combat medics (WAR).

4.2 Materials and Methods

4.2.1 Chemicals and reagents

Poly(styrene sulfonic acid) (PSSA) was purchased from Polysciences, Inc. Dulbecco’s phosphate buffer saline, Pyrrole monomer (reagent grade 98+%), 4-(3-Pyrrolyl)butyric acid (PyBA), Glucose oxidase (GOx, E.C. 1.1.3.4 from Aspergillus niger), Lactate oxidase (LOx, 1.13.12.4 from Pediococcus sp), β-D(+)glucose, Lithium lactate, and all other common solvents were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Pyrrole monomer was purified by double passage through an alumina silicate column. Solutions were prepared in deionized water prepared by purifying distilled water through a Milli-Q® plus (Millipore Inc.) ultrapure water system. The glucose stock solutions of 1.0 M and 3.0 M were prepared and allowed to mutarotate overnight.
4.2.2 Biosensor components

The MDEA 5037 transducers were acquired from ABTECH Scientific, Inc., Richmond, Virginia. The 8100-K1 fixed frequency wireless dual potentiostat system was purchased from Pinnacle Technology (Lawrence, KS). The kit contained the Pinnacle 8151 wireless dual potentiostat, the voltage programmer, and a receiver base station (model 8106) with USB cables. Software for data acquisition (PAL) was also included in the kit.

4.2.3 Cleaning of transducers

The MDEA 5037 transducers were first ultrasonicated in DI-water, IPA, and DI-water for 5 minutes each. Next they were placed in a UV-ozone cleaner (Boekel Industries) and irradiated for 10 minutes followed by 1 minute of ultrasonication in IPA. Transducers were then immersed in PBS buffer, made the working electrode of a three electrode electrochemical cell and electrochemically cleaned by sweeping the potential between 0 to -1.2 V (vs. Ag/AgCl, 3 M KCl) at 100 mV/s for 40 cycles. Transducers were finally thoroughly rinsed in flowing DI-water before use.

4.2.4 Conferring biospecificity to MDEA 5037 transducers

Working electrode arrays of MDEA 5037 transducers were first seeded with a thin electropolymerized film of PPy. This adherent film was formed with an impressed potential of 750 mV vs Ag/AgCl (3M KCl) using a charge density of 10 mC/cm² from an aqueous solution of 0.2 M Py and 0.05 M (repeat units) PSSA. Biospecificity was then conferred to the working electrode arrays through further electropolymerization in solutions containing 0.175 M Py, 0.025 M PyBA and 1 mg/mL of GOx or LOx prepared
in DI-Water with pH adjustment to 4.5-5.5 using 1 M NaOH. Electropolymerization for entrapping enzymes proceeded at 850 mV vs Ag/AgCl (3M KCl) for 100 mC/cm². Before use, enzyme-modified microelectrodes were over-oxidized (OO-PPy) by repeatedly cycling the electrode in PBS (100 mM, pH 7.2) between -200 to 1300 mV vs. Ag/AgCl (3M KCl) for 40 cycles.

**4.2.5 In vitro biosensor calibration prior to in vivo implantation:**

Biotransducers were interfaced with the 8151 wireless potentiostat via a custom connector. Two electrode electrochemical detection of glucose or lactate was utilized with the on-board working electrode array serving as the working electrode and the on-board counter electrode serving as the shorted counter and reference. Bias potential of 0.65 V was programmed into the 8151 and applied to the working electrode array of the MDEA 5037 with respect to the onboard counter electrode. To ensure functionality prior to implantation an *in vitro* calibration of MDEA 5037s were performed no longer than 8 hours prior by placing biotransducers in PBS buffer at 37 °C followed by standard glucose and lactate infusions. Steady state amperometric current produced was measured and the sensitivity of the device was determined from the slope of the calibration plot. *In vitro* calibrated sensitivity ($S_o$) is determined by the ratio of the resulting change in current ($I$) to change concentration of analyte in buffer ($G_o$ for glucose, $L_o$ for lactate) in units of µA/mM/cm². Time dependent estimations of intramuscular glucose, $G_o(t)$ and lactate, $L_o(t)$ were made based on this reference sensitivity after implantation of the device. The kinetic parameters were determined using Lineweaver-Burk (LWB) analysis of biosensor amperometric response. The apparent Michaelis–Menten constant $K_{Mapp}$ and
the maximum current response, $I_{\text{max}}$, were calculated from the slope and the intercept of the Lineweaver–Burk plots [227, 228]. Limits of detection were calculated by dividing 3*STDev of the steady-state, blank solution current response by the calculated sensitivity.

4.2.6 Implantation of biotransducers into trapezius muscles of rats

Sprague-Dawley rats weighing between 250-450 g were shaved, sterilized and placed under anesthesia on a warming pad. An incision was made above the trapezius muscle on the back to expose the fascia and muscle. Fascia was spread apart using blunt-tipped scissors to expose the muscle. A small pocket was formed by spreading the muscle tissue apart using microtweezers. The MDEA 5037 was carefully inserted into the pocket by hand. The muscle tissue was closed over working region of the biotransducer and secured with a suture, Figure 4.4(A). Fascia was secured over the muscle tissue with a suture and the wound was closed with surgical staples, Figure 4.4(B). After implantation the MDEA 5037 was interfaced with the 8151 potentiostat and allowed to reach steady state for an hour. The rat was observed during this time to ensure stability.

![Figure 4.4](image.png)

**Figure 4.4.** Sprague-Dawley rat under anesthesia with A) the MDEA 5037 biotransducer implanted into trapezius muscle and secured into place with a suture, and B) the incision closed and secured around the MDEA 5037 with surgical staples.
4.2.7 *In vivo biosensor sensitivity and response time to bolus infusion of analytes:*

After biosensor response reached a steady state baseline measurement of intramuscular analytes, measurements of blood analytes from the saphenous vein were made. Blood glucose was measured using a test strip based Alpha TRAK glucose meter (Abbott Laboratories). A bolus of solution consisting of 3 M glucose and 0.4 M lactate prepared in 1X PBS (pH = 7.4) was infused (up to 0.5 mL) through the tail vein. Interstitial analytes were measured for up to 3 hours. Additional blood analyte measurements and bolus infusions were made at 30 min intervals. Changes in amperometric response deviating from steady state were observed after the bolus injection of glucose and lactate into venous blood. Estimated interstitial analyte responses measured by biosensors calibrated in vitro were compared to basal analyte concentrations in the blood. Interstitial concentrations of glucose in non-diabetic rats have been shown to have a good 1:1 correlation to plasma glucose concentration at the basal state [289]. Sensitivity was determined based on *in vivo* responsiveness to exogenously changing levels of glucose and lactate. Clarke error grid analysis was performed to compare interstitial and blood analytes [290]. Rats were subsequently euthanized after procedures and the trapezius muscle surrounding the biotransducer was resected in order to safely recover biotransducers.

4.2.8 *Mechanisms of failure and functional life time of biotransducers*

Resected biotransducers were characterized *in vitro* to assess the functionality and lifetime of the device *in vivo*. Biosensors may cease to function as intended due to
component-based and biocompatibility-based failures [291]. The following mechanisms of failure for the explanted biosensors were analyzed: lead detachments, electrical shorts, component delamination and degradation, biofouling, electrode passivation, and fibrous encapsulation. Upon explantation the base current, sensitivity and response time of biosensors was evaluated in PBS buffer. Resected biotransducers were considered functional if sensitivity and maximum current are maintained non-significantly different from freshly prepared sensors, can detect the physiologically relevant range of glucose or lactate, and have a response time of approximately 1 minute.

4.3 Results and Discussion

4.3.1 Performance of freshly prepared lactate biosensors

Biotransducers were characterized immediately after their preparation and again immediately after being resected from the trapezius muscle. Lactate biosensors using lactate oxidase, an FMN-based enzyme, were prepared in the same fashion as glucose biosensors using glucose oxidase, an FAD-based enzyme. Lactate sensors were successful in producing a dose response, but did not produce adequate current densities in response to analytes in vitro and were therefore not considered for in vivo analysis. Dose response to lactate and biosensor performance parameters are shown in Figure 4.5 and Table 4.1. The lactate sensors had a good sensitivity of 0.36 ±0.15 µA/mM/cm². However, there was insufficient current density generated to produce a meaningful dose response using the Pinnacle 8151 wireless potentiostat. Response lactate was linear for nearly the entire range of interrogated concentration with a linear dynamic range of 6.6 ±0.9 mM. Based on the observed I_max and K_Mapp the lactate biosensors did not adhere well
to LWB analysis in addition to seeing the dose response having a linear trend. Given the
detection limits of $7.9 \pm 5.6$ mM, lactate biosensors could only accurately discern changes
in interstitial lactate concentration larger than that which is clinically relevant
(hyperlactatemia $\approx [\text{lactate}] > 4$ mM) [65, 66].

Figure 4.5. Dose response of lactate MDEA 5037 biotransducers at 650 mV bias working electrode array
versus on-board counter electrode after being freshly prepared (PBS buffer, pH = 7.2, 25 °C).

Table 4.1. Dose response (650 mV bias, PBS, pH = 7.2, 25 °C) performance parameters for lactate
biotransducers freshly fabricated ($\pm$ standard deviation is shown).

| MDEA 5037, Pt|PPy(PSSA)|p(Py-co-PyBA)-LOx [E] = 1.0 mg/mL | Freshly prepared biotransducers (n = 3) |
|--------------|-----------------------------------|--------------------------------------|
| Sensitivity ($\mu$A/mM/cm$^2$) | 0.36 $\pm 0.15$ |
| Response time (s) | 86 $\pm 26$ |
| Detection limit (mM) | 7.9 $\pm 5.6$ |
| $K_{Mapp}$ (mM) | -9.1 $\pm 15.8$ |
| $I_{max}$ ($\mu$A/cm$^2$) | -1.9 $\pm 5.8$ |
| Linear range (mM) | 6.6 $\pm 0.9$ |
4.3.2 Performance of freshly prepared and resected glucose biosensors

Glucose biosensors successfully produced dose response curves to analytes in vitro. The dose response of glucose sensitive MDEA 5037s are shown in Figure 4.6. The changes in glucose biosensor performance parameters are summarized in Figure 4.7 and Table 4.2. The MDEA 5037 biotransducers were implanted in muscle tissue for up to 3 hours. The overall performance of resected biotransducers was diminished compared to when they were freshly prepared which was expected. Sensitivity decreased from 0.68 ±0.40 to 0.22 ±0.17 µA/mM/cm² (p = 0.08), response time increased from 41 ±18 to 244 ±193 s (p = 0.08), detection limits increased from 0.05 ±0.03 to 0.27 ±0.27 mM (p = 0.17), K_{Mapp} increased from 7.3 ±3.4 to 74 ±130 mM (p = 0.35), I_{max} decreased from 11.7 ±6.5 to 8.8 ±9.3 (p = 0.30), and linear range increased from 7.2 ±5.9 to 14.1 ±11.6 (p = 0.34). Biotransducers were subject to blood and protein adsorption as well as the acute inflammatory response during their relatively short implantation period. The dramatic increase in response time and K_{Mapp} is likely due to proteins adsorbing from the interstitial fluid to the biotransducer surface. Protein adsorption has been shown to have a negative influence on glucose diffusion for implantable and continuous biosensor systems [138, 292]. No deterioration of the MDEA 5037 transducer was observed and none of the resected biotransducers had a spurious response indicating that neither instrumentation problems nor transducer compromises were causing the changes in performance. Limits of detection were maintained at adequate levels to discern changes in glucose using the biosensor system. Production of hydrogen peroxide has been identified as the primary agent for short-term enzyme degradation in vivo [293]. The lack of significant change in
maximum current shows that the original enzyme activity is still present in the system after short-term use *in vivo*, supporting these previous findings. Overall the MDEA 5037 biotransducers maintained their activity after short-term implantation with respect to sensitivity, maximum current, range of response and response time.

**Figure 4.6.** Dose response of glucose MDEA 5037 biotransducers after being freshly prepared and after resection from rat trapezius muscle (650 mV bias, PBS buffer, pH = 7.2, 25 °C, ± standard deviation is shown).

**Figure 4.7.** Glucose biosensor performance parameters of freshly prepared and resected biotransducers (650 mV bias, PBS buffer, pH = 7.2, 25 °C, n = 4, ± standard deviation is shown).
Table 4.2. Dose response performance parameters for biotransducers freshly fabricated and resected from trapezius muscle after c.a. 3 hours of implantation (650 mV bias, PBS, pH = 7.2, 25 °C, ± standard deviation is shown).

| MDEA 5037, Pt|PPy(PSSA)|p(Py-co-PyBA)-Gox [E] = 1.0 mg/mL | Freshly prepared biotransducers (n = 4) | Resected biotransducers (n = 4) |
|----------------|-----------------------------|---------------------------------|---------------------------------|
| Sensitivity (µA/mM/cm²) | 0.68 ±0.40 | 0.22 ±0.17 |
| Response time (s) | 41 ±18 | 244 ±193 |
| Detection limit (mM) | 0.05 ±0.03 | 0.27 ±0.27 |
| K_{Mapp} (mM) | 7.3 ±3.4 | 74 ±130 |
| I_{max} (µA/cm²) | 11.7±6.5 | 8.8±9.3 |
| Linear range (mM) | 7.2±5.9 | 14.1±11.6 |

4.3.3 In vivo response of MDEA 5037 biosensors to exogenously changing analytes

Steady state current was allowed to be reached for one hour after implantation of the biotransducer into the trapezius muscle. Three MDEA 5037 biosensors were tested in vivo (MDEA-1, MDEA-2 and MDEA-3). The amperometric responses of the sensors in vivo are shown in Figures G1, G2 and G3. MDEA-1 and MDEA-3 were prepared having a glucose sensitive cell (Channel 1) and a lactate sensitive cell (Channel 2). Response of Channel 2 for these biotransducers was omitted for clarity. MDEA-2 utilized two glucose sensitive cells (Channel 1 and 2) to compare differences in response to glucose on the same biotransducer. Intramuscular glucose via the biosensor was compared to venous blood glucose from the saphenous vein measured with the Alpha TRAK. Interstitial glucose has been shown to lag blood glucose by approximately 5 minutes [294]. Over the implantation time periods there was sufficient time for interstitial
glucose to follow blood glucose measurements. The first sensor, MDEA-1, came to a stable base-line current, and interstitial glucose concentrations did not vary much between 1.91-2.23 mM, Figure 4.8 and Table 4.3. This seemed reasonable as blood glucose did not vary, remaining between 5.49-5.88 mM. One of the sensors (MDEA-2, Channel 2) showed a sustained increase in interstitial glucose after tail vein infusions of glucose, Figure 4.9 and Table 4.4. For the case of MDEA-2, both channels were sensitive to glucose. However, only one of the channels (Channel 2) showed a change in interstitial glucose while the other (Channel 1) did not. MDEA-3 showed a decreasing trend of interstitial glucose concentration with a rapid increase and subsequent fall within minutes after a tail vein infusion, Figure 4.10 and Table 4.5. Interstitial and blood glucose concentrations in anesthetized rats at the basal level are typically statistically similar, but insulin has been shown to cause up to a 60% difference between the two compartments within 30 minutes [295]. Glucose concentrations have been shown to change significantly faster in the blood of anesthetized rats with a rate of 6.8 ±2.0 mg/dl/min compared to subcutaneous fluid having a rate of 3.9 ±1.3 mg/dl/min [295].
Figure 4.8. MDEA-1 Amperometric response at 650 mV bias to glucose *in vivo* in the trapezius muscle of a male Sprague-Dawley rat of 412 g (infusions were of 3 M glucose in PBS).

Table 4.3. MDEA-1 Biosensor and saphenous glucose comparison.

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Saphenous glucose (mM)</th>
<th>Biosensor glucose Ch1 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4360</td>
<td>5.49</td>
<td>2.23</td>
</tr>
<tr>
<td>6330</td>
<td>5.72</td>
<td>1.91</td>
</tr>
<tr>
<td>8380</td>
<td>5.88</td>
<td>1.92</td>
</tr>
</tbody>
</table>
Figure 4.9. MDEA-2 Amperometric response at 650 mV bias to glucose \emph{in vivo} in the trapezius muscle of a male Sprague-Dawley rat of 408 g (infusions were of 3 M glucose in PBS).

Table 4.4. MDEA-2 Biosensor and saphenous glucose comparison.

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Saphenous glucose (mM)</th>
<th>Biosensor glucose Ch1 (mM)</th>
<th>Biosensor glucose Ch2 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3531</td>
<td>10.21</td>
<td>0.67</td>
<td>5.01</td>
</tr>
<tr>
<td>4837</td>
<td>9.93</td>
<td>0.74</td>
<td>5.97</td>
</tr>
<tr>
<td>5982</td>
<td>10.88</td>
<td>0.71</td>
<td>7.95</td>
</tr>
<tr>
<td>7111</td>
<td>11.27</td>
<td>0.67</td>
<td>8.54</td>
</tr>
</tbody>
</table>
Figure 4.10. MDEA-3 Amperometric response at 650 mV bias to glucose in vivo in the trapezius muscle of a female Sprague-Dawley rat of 265 g (infusions were of 3 M glucose in PBS).

Table 4.5. MDEA-3 Biosensor and saphenous glucose comparison.

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Saphenous glucose (mM)</th>
<th>Biosensor glucose Ch1 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3736</td>
<td>13.65</td>
<td>3.55</td>
</tr>
<tr>
<td>5344</td>
<td>19.65</td>
<td>0.44</td>
</tr>
<tr>
<td>6381</td>
<td>16.26</td>
<td>1.28</td>
</tr>
</tbody>
</table>

4.3.4 Clinical accuracy of the MDEA 5037 wireless biosensor system

The correspondence between the two systems is summarized in Figure 4.11. Intramuscular glucose measurements were consistently lower than blood glucose measurements. Clarke’s error grid analysis was utilized to assess the clinical significance of difference between the MDEA 5037 biosensor and the Alpha TRAK reference, Figure 4.12. Zone A represents glucose values that deviate no more than 20% of reference values, and the values within this range are considered to be clinically exact. Zone B are
considered to be benign errors. Values in zones A and B are considered clinically acceptable, whereas values in zones C-E are considered dangerous [296]. The MDEA 5037 biosensors had a total of 0 points in zone A, 7 points in zone B, 1 point in zone C, 0 points in zone D, and 6 points in zone E. Although none of the biosensors agreed exactly with the reference, the MDEA 5037 and Pinnacle 8151 wireless biosensor system had a clinically acceptable accuracy of 50%. Background current could not be fully accounted for with these biosensors due to both electrochemical channels being used for simultaneous analyte detection. Accuracy of continuous monitoring can be improved by correcting for background current [297]. Background current stability of biosensors is known to drift in vivo [298]. The background current of MDEA 5037 biotransducers can increase by two orders of magnitude from 0.5 nA to 20 nA after a week of storage in 0.1 M PBS (pH = 7.2) buffer at 25 °C (data not shown). Future studies will need to take into consideration background current immediately prior to implantation. Electroconductive hydrogel modified biotransducers will also be utilized in future studies, which will likely have better stability in vivo compared to just polypyrrole modified biotransducers. The biosmart hydrogels have been shown to mitigate protein adsorption [131] which may lead to better short-term performance in vivo. The inclusion of hydrogels will be necessary for the protection of enzymes and mitigation of fibrous encapsulation for long-term implantation of biosensors.
Figure 4.11. Comparison of intramuscular (MDEA 5037 biosensor) to venous blood (Alpha TRAK handheld) glucose measurements.

Figure 4.12. Clarke’s error grid analysis of implanted MDEA 5037 biosensors with respect to hand-held glucose monitor Alpha TRAK measuring venous blood as a reference.

4.4 Conclusions

The MDEA 5037 and Pinnacle 8151 wireless biosensor system was successfully prepared and characterized in vivo. Both glucose and lactate biosensors were prepared and interrogated in vitro prior to implantation. Lactate biosensor performance was insufficient for characterization in vivo as the limits of detection were not small enough.
Glucose biosensors performed as expected based on previous characterization *in vitro*. MDEA 5037 Biosensor systems maintained functionality after 3 hours of implantation in the trapezius muscle of Sprague-Dawley rats. Non-significant decreases in sensitivity and maximum current were observed after biotransducers were resected. Protein adsorption onto the MDEA 5037 biotransducer surface after short-term use *in vivo* is likely the cause for dramatically increased response time and $K_{\text{Mapp}}$. The biosensor system had a clinically acceptable accuracy of 50% when comparing interstitially measured analytes to a reference hand held glucose monitor. To improve both short-term and long-term performance *in vivo* the biotransducers will be coated in hydrogel in the future to mitigate protein adsorption. Background current will also need to be taken into consideration for improving *in vivo* accuracy of MDEA 5037 biosensor systems. This may be accomplished by either utilizing sensors non-specific to analytes during measurement or measurement of steady state current in an interstitial fluid/buffer immediately prior to implantation.
CHAPTER FIVE: FUTURE WORK AND COMMENTARY ON IMPLANTABLE AMPEROMETRIC BIOSENSORS

5.1 Introduction

This last chapter highlights areas of ongoing research with respect to implantable biosensors. There are a number of avenues for future work and research when it comes to implantable devices and specifically biosensors like the MDEA 5037 system. The primary goal for future work on implantable amperometric biosensors will be continued investigation of methods and approaches to influence in vivo performance. Other important avenues include developing oxygen independent systems, determining the usefulness of biosensor vital signs for healthcare professionals, integration of biosensor vital signs with established physical vital signs, systems integration, and internal calibration.

5.2 Improving the current generation of implantable amperometric biosensors

For an indwelling biosensor, such as an intramuscularly implanted biosensor, the temperature of the surrounding tissue bed will have influence on the amperometric current response based on the Arrhenius relationship [299]. For a biosensor used in the monitoring of vital signs of a trauma patient, onset of events such as hypothermia will have ramifications for the performance of the biosensor. Physiological and metabolic changes in the body will also accompany changes in temperature. Temperature has shown to have effects on the catalytic activity of the enzymes, permeability of analytes
through substrates, and the water solubility of oxygen [300]. Future iterations of implantable biosensors will need to account for temperature and thereby reduce uncertainty in the basis for changes of the amperometric response.

Signal processing procedures necessary for implantable sensors have been outlined and include low-pass filtering, DC offset adjustment, and amplification [70]. Raw signals from biosensors have been considered as a convolution of an analyte-specific function and a series of interference functions such as temperature changes and mechanical instabilities of the implanted device [301]. Algorithms have been developed for real time management of raw data, providing compensation electrical and mechanical perturbations, temperature fluctuation, enzyme inactivation, and nonlinearity of analyte dose response [301]. Interference functions that are mechanical or electrical in origin, are filtered using a process known as clipping, which is the suppression of rapid signal variations that are faster than the fastest physiological changes in analyte concentration [301].

Another approach to address interferences is to use interrogation potentials that are displaced from the redox signals of the interferents. This is accomplished by the use of surface confined electrocatalysts such as poly(neutral red) / flavin adenine dinucleotide (FAD) hybrid films [188], lead pentacyanonitrosylferrate [302], Prussian Blue and its hybrids [303] or molecular redox mediators. Decreases in biosensor signal are expected to occur over time (drift). For any such decrease in signal over time, fifty percent of the total decrease can be attributed to inactivation of enzyme, thirty percent attributed to leaching of mediators, and twenty percent attributed to decreases in background current.
leading to only an apparent loss of signal [300]. Hence emphasis must be placed on reducing enzyme denaturation, eliminating the use of mediations, and eliminating background currents.

Prussian blue (PB) is a well-known, highly stable, highly selective catalyst for hydrogen peroxide reduction. Reduction of H$_2$O$_2$ can be performed at a PB modified surface using potentials as low as 0 V vs Ag/AgCl with better effectiveness than peroxidase enzyme modified surfaces [304]. Utilizing such a low potential would mitigate many interfering responses. Heterogeneous rate constants for peroxide reduction of 0.015-0.02 cm/s have been reported [305]. In comparison the kinetic constant for peroxide oxidation at platinum electrodes is three orders of magnitude lower than this [306]. Prussian blue and polypyrrole can both be electrodeposited onto electrodes [307]. This technique will be explored in future iterations of the ECH-modified MDEA 5037 biotransducer to maximize response to hydrogen peroxide generated by glucose oxidase and lactate oxidase. Underpotential deposition of platinum and gold onto MDEA 5037 electrodes to form nanoparticles will also be explored. Gold and platinum nanoparticles can be used to achieve similar catalytic oxidation and reduction of hydrogen peroxide at low operating potentials [308, 309].

5.3 Optimizing electroconductive hydrogels for enzyme stability in vivo

Enzyme stability in a biosensor application can be defined as the reproducibility of analytical response or signal to analyte (substrate) over a period of time. Factors influencing signal reproducibility include the exact activity of immobilized enzymes [310], possible enzyme denaturation during immobilization onto electrodes [311],
enzyme degradation over time by reactive oxidative species [312], and protease degradation of the immobilized enzyme [313]. It has been well established that stability of enzymes will be directly affected by the immobilization technique used and the microenvironment into which the enzymes are immobilized [314]. Conventional immobilization techniques include physical adsorption [182], biomolecule entrapment with or without covalent tethering such as within a hydrogel [2, 183, 184], covalent cross-linking using homo- and hetero-bifunctional cross linkers [186], and direct covalent bonding to the electrode’s surface [185]. Entrapment is one of the most appropriate techniques for implantable devices as it is safe and easy to perform, requires little to no toxic chemicals, and maintains enzymes in a confined area near an electrode without chemically perturbing their native structure [315].

To address the needs of the system, we have developed soft, condensed, biomimetic but otherwise inherently electronically conductive materials to address the challenge of interfacing solid state devices with the electronics of the body, which is predominantly ionic [316]. These electroconductive hydrogels offer the potential for biocompatibility while sustaining electrical fidelity across the biological-to-solid state interface. Electroconductive hydrogel coating of biotransducers has shown to be beneficial for mitigating protein adsorption, fibrous encapsulation, enzyme stability and control of transport properties of biomolecules. The current drawback to using ECH modified systems is low current densities due to diffusion limitation of analytes to the electrode surface. While sophisticated potentiostat technology can be utilized to circumvent these issues by implementing low current modules, it becomes more difficult
to produce a small, wireless system with the same amplification and filtering capabilities. Increasing the degree of hydration by lowering the overall crosslink density of hydrogels to improve current densities will be a future work in optimizing ECH-modified biotransducers. This may serve to effectively increase the free water content of the system to better facilitate enzyme entrapment, and decrease diffusional barriers and tortuosity. The stiffness of the ECH will also need to be considered during the optimization as it will have an effect on cell viability and proliferation.

5.4 Second generation biosensors and use of mediators for electron transfer

Mediators are molecules, moieties of polymers, complexes, and inorganic layers that mediate electron transfer between the redox active co-factors of immobilized enzymes and the electrode. A key requirement of the mediator is to provide a high electron transfer rate constant ($k_{\text{ET}}$) with the prosthetic group of the enzyme as well as with the electrode in order to obtain high currents, lowered limits of detection and increased sensitivity. As a result, the necessary applied potential can be reduced to values where fewer potentially interfering electroactive species are non-specifically oxidized or reduced. Other requirements for mediators are that they be suitably soluble in aqueous media, have well-characterized reversible redox behavior, possess chemical stability in both the oxidized and the reduced forms, be of an appropriate redox potential to allow mitigation of the effect of interfering substances, be un-reactive with the targeted enzyme substrate, its product or other substances, be insensitive to pH and ionic strength effects, and most of all be non-cytotoxic. Mediators are crucial for implantable sensors where oxygen levels are significantly reduced (hypoxia) and/or highly variable, such as during a
state of tissue ischemia. Oxygen is a highly competitive molecule against other mediators for the oxidation of reduced enzymes. Glucose oxidase has a turnover rate of approximately 1000 s\(^{-1}\) in the presence of oxygen [138].

Molecular mediators such as ferrocene, and electroactive polymers such as poly(vinylferroence) have been explored but found wanting. Polymers based on Os\(^{2+/3+}\) complexes 2,2’-bipyridine (bpy) of type [Os(2,2’-bipyridyl)2LL’] and [Os(4,4’-dimethyl-2,2’-bipyridyl)2LL’] (where LL are ligands) form stable, reversible, redox couples and have shown tremendous promise but have not been fully evaluated \textit{in vivo} [317]. Electroconductive polymers such as polypyrrole, polythiophene and polyaniline have also been used as mediators wherein electrons are transferred between the enzyme redox center and the polymer chain allowing for bias potentials as low as 0.0 V \textit{vs.} Ag/AgCl to be used [318]. However, peroxide generated by enzymes will overoxidize the polypyrrole network over time, diminishing its electroconductive and mediating properties. Future work will include tethering of osmium and ruthenium complexes into hydrogel networks to improve oxygen independence of biosensor systems. The intramuscular biocompatibility of mediator loaded hydrogels will also need to be examined.

\textbf{5.5 Third generation biosensors and direct electron transfer}

Generation III biotransducers allow direct electron transfer between the redox active co-factor of the oxidoreductase enzymes and the underlying electrode. In this respect they obviate the need for molecular oxygen and/or molecular mediators. Carbon nanotubes (CNTs) and graphene with their high chemical stability, high surface area, excellent mechanical properties, and high electrical conductivity are now being
extensively investigated for the construction of nanobiosensors [319-321]. The electrical and electronic properties of CNTs enhances electron transfer, making them suitable for the preparation of electrode materials and for use in chemical and biological sensors [322, 323]. The use of CNTs for biosensor electrode applications has been demonstrated [320, 324] and their application towards electrochemical sensors and biosensors has gained significant momentum [320, 325]. Covalent functionalization changes the chemical properties of nanotubes dramatically. However, this has allowed covalent and ultrasonic non-covalent conjugation [326] to redox proteins and so allows for the efficient immobilization of glucose oxidase; allowing the CNTs to simultaneously act both as an immobilization matrix and as a redox mediator or electrocatalyst [327, 328]. Direct electron transfer has been demonstrated, however, the dose response to glucose for these systems has been less efficient in deoxygenated conditions compared to O2-saturated conditions [329, 330]. Oxygen independent systems, while currently less efficient than an oxygen dependent systems, is more valuable especially for trauma monitoring applications in hypoxic tissues where oxygen tension is very low. Although there have seen significant research to produce glucose biosensors with reliability for in vitro or in vivo applications [331, 332], the recent availability of nanostructured electrodes that allow direct bio-electrochemical reactions with the buried FAD [330] is promising for the development of reagentless biosensors, independent of oxygen tension, for eventual clinical use. In view of their above mentioned properties, carbon nanotubes are under intensive investigation as potential building blocks for implantable amperometric biosensors.
Electrodes used for carbon nanotube immobilizations are typically high surface area such as electrospun fibrous metal or glassy carbon [329, 330]. Transducers like the MDEA 5037 utilize platinum and gold for their working electrodes which can be modified to produce a variety of surface topographies and chemistries. Future work may encompass depositing carbon nanotubes onto the MDEA 5037 working electrode surfaces for immobilizing carbon nanotubes to be used for conjugation with enzymes. For polypyrrole immobilized single-walled carbon nanotubes (SWCNT) via electropolymerization, preliminary studies have shown that either nanotubes and/or GOx-SWCNT conjugates are providing conduction between the solution and platinum electrode through the nano-thin polypyrrole layer, Figure 5.1. Approximately 80% of the original electrode area is blocked by PPy-GOx whereas only 25% of the original electrode area is blocked by PPy-GOx-SWCNT. Blocking and insulation of the electrode to Ferrocene Carboxylic acid (FeCOOH) by polypyrrole was expected [12]. Having immobilized SWCNTs onto MDEA 5037 transducers there is now potential for direct electron transfer between subsequently immobilized enzymes and electrodes.
**Figure 5.1.** (Left) Cyclic voltammetry of bare platinum, PPy-GOx and PPy-GOx-SWCNT systems in 1 mM FcCOOH in PBS buffer (pH = 7.2) at 100 mV/s. (Right) Peak anodic current as a function of scan rate (error bars show standard deviation).

### 5.6 Systems integration

Implantation is motivated by the need to allow the patient or subject the freedom of motion without being confined to an instrument; implantation allows the subject to take the instrument with them. Central to an implantable amperometric enzyme biosensor is its potentiostat. However, for implantation, the potentiostat is closely associated with two-way telemetry and communications. Three general formats are being pursued. The first is an implantable but otherwise tethered biotransducer with externally located power, electronics and communications components with the external components being mounted outside but on the subject’s body [166]. The second is a fully integrated discrete but otherwise fully implanted device [333] and the third is an application specific integrated circuit (ASIC) where all components are likewise fully implanted [334]. Representative examples of these three formats are shown in **Figure 5.2.** Simple electrochemical techniques such as fixed-potential amperometry have been successfully demonstrated. However, further improvements may be made by including additional...
electrochemical techniques such as sweep and pulse voltammetry. Improved filtering and amplification capabilities of will enable detection of low current densities. The ability to wirelessly program potentiostats will also be necessary for indwelling devices.

![Figure 5.2](image)

**Figure 5.2.** Examples that illustrate the three general formats for implantable biosensor systems. A) The tethered biotransducer with externally located power, electronics and communications components. B) The fully integrated discrete but otherwise fully implanted biosensor system. C) The application specific integrated circuit (ASIC).

### 5.6.1 Device footprint

Cutting-edge, low-profile (small footprint) wireless potentiostats such as the Pinnacle 8151 have been successful in producing implantable wireless glucose biosensors. Decreasing the overall footprint of biosensor systems will be necessary in future iterations. The footprint of an implantable amperometric biosensor is defined by the volume of space the device takes up and represents the judicious balance between use
of space and functionality. Ideally, an implantable biosensor system should be as small as possible, have very low power consumption, and be able to wirelessly transmit data and be remotely programmed while implanted within living tissues. Major electronic components include the front-end potentiostat, power modules, microcontrollers, random access memory, flash memory, RF transmitters, and analog-to-digital converters (ADC)\[335\]. Miniaturized potentiostats have been developed which contain microprocessors, ADC, gain and low-pass filters [153, 336-343]. Continued innovations in potentiostats are needed. A miniature conformal antennae for implantable telemetry applications has been developed to help minimize footprint wherein the entire package of the implantable Bio-Nano-Sensor system is approximately 2477 mm$^3$ (≈2.5 mL) in volume [344]. Bio-Nano-Sensors with electrochemical front-ends have been developed for continuous monitoring of alcohol [333], glucose, lactate, glutamate, and ATP [345]. Subdermal identification (RFID- Radio Frequency Identification) chips such as the VeriChip® are approximately 12-14 mm$^3$, approximately two orders of magnitude smaller. However, while application specific integrated circuits (ASIC), these devices at not equipped for bioanalytical measurements. The unequivocal ambition of in vivo bioanalytical and clinical chemistry is to develop and deploy physiological status monitoring biochips of similar footprint, along the lines of a large grain of rice, Figure 5.3. The technical and financial feasibility of this should become more apparent when considering Moore’s law of increasing number of transistors per unit area of integrated circuits.
5.6.2 Wireless architecture

Wireless biosensor systems are important for continuous monitoring of analytes at the molecular level in both animal models and human patients [346]. Wireless implantable biosensor systems must perform the following functions: i) manage power for all components of the system, ii) acquire and condition analog signals, iii) digitize analog signals, iv) store readings and operational parameters, v) support communication to and from a base station, and vi) be able to switch between an active and low-power or sleep state [335]. The Texas Instruments (TI) MSP430 ultra-low power and mixed signal microcontrollers are readily matched with AD converters, wireless transceivers and application specific circuitry to provide a valuable development platform. The CC1110 (TI/Chipcon) is a small, surface mounted integrated circuit (IC) that been determined to
be one of the best solutions for a discrete implantable system as it has a fully 
implemented microprocessor, RF transceiver, and ADC [335]. A low-power, high-speed, 
ultra-wideband (UWB) transmitter in a wireless transmission test platform for 
implantable biosensors has been developed. Occupying 420 μm², the integrated 
transmitter consists of a compact pulse generator and a modulator [347]. The transmitter 
generates 1ns pulse widths at pulse rates of 90-270 MHz and achieved 14 Mbps data rate. 
At a 50% data duty cycle, power ranged from 10-21 mW over a transmission distance of 
3.2-4.0 meters. The site of implantation and the environment of operation of the 
biosensor will determine the requirements of the RF transceiver in terms of frequency of 
operation. The MAX4039 (Maxim integrated products) is a commercially available IC 
that has already been utilized in implantable biosensors for animal modeling [345]. Novel 
low power IC designs and power harvesting circuits have also been employed in some 
select case studies [348]. New wireless architecture enables data processing on hand-held 
devices such as mobile phones and by cloud computing. The ability to save history and 
trends of patient physiologic status can facilitate powerful retrospective analysis with 
respect to patient outcomes. This will come with issues of latency, response time and 
wireless security that will need to be considered.

5.6.3 Power requirements

Implantable amperometric biosensor systems, because of their analog front ends 
and the need for mixed signal electronics, processing, and communications, are power 
demanding devices. Acknowledged power sources are batteries, inductively coupled 
power sources, or an internally sustainable source of energy such as a biofuel cell.
Batteries have thus far failed to deliver the combined power density and footprint required of fully implantable bioanalytical devices. Inductively powered sources based on novel stacked spiral antenna designs are beginning to emerge and are providing sufficient power to meet the demands of implantable bioanalytical devices [349]. Micro-biofuel cells based on enzyme reactivity represent a clear sustainable possibility [350]. Facile methods to develop lithium-ion batteries comprised of anode materials of SnO$_2$-nanocrystal/grapheme-nanosheets have been explored for potential use in biosensor applications, but are still in the nascent stages of development and optimization [351]. In any case, power management will become central to the performance of wholly implantable biosensors.

### 5.7 Usefulness of data to caregivers

The format for presenting data can affect decision making [352, 353]. In general, the faster directed resuscitation efforts are performed the better opportunity patients have for survival [354]. While under development, it is important to understand and delineate the suitability and applicability of an implantable device to the real world need. Determining how to appropriately present data in a fast-paced, data-rich environment requires judicious attention to the decision support needs and the context of use for each group of end users (e.g. EMS, emergency physician, surgeons, RNs). It will be paramount to identify i) what physiological status data, if any, is currently available to all healthcare professionals (e.g. EMTs, emergency physicians, trauma surgeons, and nurses) that care for the trauma patient from site of accident through recovery, ii) the key physiological parameters that are useful in making patient care decisions, iii) the data
needs during transition from prehospital to hospital based care between ambulance and emergency department/trauma center, operating theater, and ICU, and iv) the variability among two admitting centers. It will also be important to identify all participants in the chain of patient custody throughout the time course of trauma. Future studies will include conducting interviews with pre-hospital personnel, nurses and physicians, and developing plans for biosensor handling between these personnel. This will be necessary in order to produce a clear chain of care that does not violate privacy guidelines or compromise the integrity of the device and associated data. The goal is to develop a display system that presents data in a way such that it supports the cognitive work (sensing and perceiving information, decision-making, problem solving, adapting, etc.) of healthcare professionals. Thus the biosensor display may act as a cognitive aid to reduce potential decision-making and communication errors, reduces decision-making time, increases system efficiency and potential survival rate of trauma patients.

### 5.8 Metabolic markers of trauma and integration with existing vital signs

It has been shown that combinations of vital signs are better at predicting trauma patient outcome and need for lifesaving intervention better than individual vital signs [355]. The Wireless Vital Signs Monitoring (WVSM) system by Athena GTX has shown that a holistic understanding of physical vital signs is more accurate in predicting outcomes than monitoring single physical events individual. These types of combination vital signs have been used to produce close-loop resuscitation systems [356]. Future work in monitoring trauma physiology will involve determining the predictive capability of
combined biosensor vital signs with well-known physical vital signs such as heart rate, pulse pressure and systolic blood pressure.

5.9 Internal calibration systems

A major challenge with indwelling biotransducers of the enzyme amperometric type is the need for and approach to in vivo calibration of the device. It is commonplace to conduct in vitro calibrations via standard additions when the sample matrix is complex. Such techniques are generally not accessible in vivo. Accordingly, in vivo amperometric biosensors must be designed to perform with a simple one-point calibration. One-point calibration is generally based on blood derived from a single finger prick. One point calibrations are inherently fraught with error and are notoriously inadequate when errors are more complex than systemic and/or random. Multi-point calibrations of in vivo biotransducers is possible by the administration of a bolus of glucose or lactate to the patient or animal subject, and is often done in animal studies and may be done in a clinical setting [9, 298]. However, this is not generally possible or recommended in the chronically monitored patent. Moreover, this approach assumes that interstitially or subcutaneously monitored analytes are at the same concentration as their systemic circulating counterparts, or at least they are consistently proportional due to transport barriers. While under homeostasis, the latter may be true, however the dynamics of the physiological changes suggest otherwise, such as during hemorrhage [3]. In order to get the most accurate interstitial analyte readings, future studies will investigate the best possible calibration method for the MDEA 5037 biosensor prior to implantation.
5.10 Conclusions

The ultimate goal of this work is to decrease the number of deaths due to trauma related hemorrhage. Enzyme based amperometric biosensors are currently the dominant *in vivo* format for the specific detection of biomolecules and are showing great promise in their ability to be translated into an advanced diagnostic platform for trauma. We are guided by critical analysis to the value of glucose and lactate as biomarkers of trauma and hemorrhage. Enhancing biosensor performance for the measurement of glucose and lactate will serve to develop this novel trauma diagnostic, and it will serve the need for sustained research of numerous other implantable devices. The small size of microfabricated biotransducers and their many possible modes of implantation open up unexplored avenues for physiologic status monitoring. Opportunities include, but are not limited to, measurement of i) lactate in the heart for predicting outcomes and endpoints of cardiovascular surgery [357], ii) acetylcholine in the brain to measure brain seizure activity [358] or observing effects of drugs on learning and memory [359], and iii) observing hepatic glycogenesis in response to hyperglycemia and hyperinsulinemia for diabetes research [360]. The potential for *in silico* modeling of biological transport phenomena between the compartments also exists. The continued integration of transducers, potentiostats, enzymes, hydrogels, physiology and nanotechnology will guarantee the sustained research and development in this field.
## APPENDIX A: Electrode Geometries for Amperometry

<table>
<thead>
<tr>
<th>Geometry</th>
<th>Disc electrode</th>
<th>Microdisc electrode</th>
<th>Microdisc electrode array</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>Current profiles</td>
<td>$i(t) = \frac{nFAD_{app}^{1/2}c^*}{\pi^{1/2}r^{1/2}}$</td>
<td>$i(t) = \frac{nFAD_{app}^{1/2}c^<em>}{2\pi^{1/2}r^{1/2}} + \frac{nFAD_{app}^{1/2}c^</em>}{2r}$</td>
<td>$i(t) = \frac{nFAD_{app}^{1/2}c^*}{\pi^{1/2}r^{1/2}} :: \frac{i}{i_e} \gg$</td>
</tr>
<tr>
<td>Applicable conditions</td>
<td>Planar electrode; semi-infinite linear diffusion</td>
<td>Planar electrode, hemispherical diffusion</td>
<td>Array of disc electrodes, multiple domains of diffusion</td>
</tr>
<tr>
<td>Steady state current</td>
<td>$i_{ss} = 4nFCD_{app}r_e$</td>
<td>$i_{ss} = 4nFCD_{app}r_e$</td>
<td>$i_{ss} = 4nFCD_{app}r_e$</td>
</tr>
<tr>
<td>Diffusion field</td>
<td><img src="image4" alt="Diffusion Field" /></td>
<td><img src="image5" alt="Diffusion Field" /></td>
<td><img src="image6" alt="Diffusion Field" /></td>
</tr>
</tbody>
</table>

**Table A.1:** Disc, Microdisc and Microdisc array geometries of implantable amperometric electrodes, their associated forms of the Cottrel’s equation and schematics of the diffusive mass transport field associated with each type of electrode.
Table A.2: Disc, Microdisc and Microdisc array geometries of implantable amperometric electrodes, their associated forms of the Cottrel’s equation and schematics of the diffusive mass transport field associated with each type of electrode.
REFERENCES


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