Advances in Glucose Sensing Techniques: Novel Non-Invasive and Continuous Electrochemical Glucose Monitoring Systems

By

Kyoung Ha Cha

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Doctoral Committee:

Professor Mark E. Meyerhoff, Chair
Assistant Professor Rodney C. Daniels
Professor Raoul Kopelman
Professor Kyung-Dall Lee
Associate Professor Stephen Maldonado
Kyoung Ha Cha
chakyoun@umich.edu
ORCID iD: 0000-0002-6898-251X

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DEDICATION

This work is dedicated to my family and my lovely wife.
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ABSTRACT

Diabetes mellitus (or diabetes) is a chronic, lifelong condition that affects the body’s ability to utilize the stored chemical potential energy found in our food. Frequent measurement and tight control of blood glucose is essential to avoiding life-threatening hyper- and hypoglycemic events and associated serious, long-term complications. In this dissertation, a novel non-invasive tear glucose measurement approach and various continuous electrochemical glucose sensor-based monitoring devices with nitric oxide (NO) release are examined and evaluated for their potential application for diabetes management. Tear glucose measurements have been previously suggested as a potential alternative to blood glucose monitoring for diabetic patients, although this approach has not been thoroughly established. In Chapter 2, the first use of commercial blood glucose test strips to measure glucose in tears is examined. Roche AccuChek test strips are shown to exhibit the low detection limit required for quantitating glucose concentration in tears. Measurements of glucose in tears from nine normal (nondiabetic) fasting human subjects using strips yielded glucose values within the range of 5–148 µM, similar to glucose measurements for human tears reported by others via LC-MS methods. Chapter 3 evaluates the origin of the high sensitivity and selectivity of the Roche test strips and demonstrates that the use of pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) in combination with a nitrosoaniline derivative as an electron transfer mediator provides the low limits of quantitation (ca. 9 µM) and enhanced selectivity achieved with these strips. In Chapter 4, the test strips are used to measure glucose
levels in tear fluids from human subjects with type 2 diabetes after fasting and then for 90 min after ingesting sugar while concurrently measuring the blood glucose values. A moderate correlation between tear and blood glucose levels is demonstrated.

Tight glycemic control helps reduce life-threatening hyper- and hypoglycemic events that can cause serious long-term complications for hospitalized critically ill patients. Therefore, the development of continuous glucose monitoring systems to quantitate blood glucose levels intravascularly (IV) could improve patient outcomes. In Chapter 5, the compatibility of nitric oxide (NO) release coatings with implantable enzymatic glucose sensors based on osmium (III/II) mediated electrochemical detection is examined for the first time. Nitric oxide (NO) is a potent inhibitor of platelet activation and adhesion. NO-releasing osmium-mediated glucose sensors are prepared using a S-nitrosothiol impregnated outer tubing and are tested in vitro in both phosphate buffer (pH 7.4) and heparinized whole porcine blood. After 3 days of continuous NO release at or above physiological levels, no negative effects on the osmium mediated electrochemical currents are observed. These results suggest that improved performance of both intravascular and, potentially, subcutaneous Os(III/II) mediated glucose sensors may be realized by incorporating NO’s well-known anticlotting, anti-inflammatory, and antimicrobial properties.
CHAPTER 1

Introduction

1.1 Current Status of Diabetes Mellitus

The World Health Organization (WHO) reports that more than 347 million people worldwide live with diabetes. Over 1.1 million deaths in 2005 were caused by diabetes, and deaths attributed to diabetes are estimated to double by 2030.\(^1\) Typically, a personal blood glucometer with single-use test strips allow diabetic patients to monitor their blood glucose level by obtaining and quantitating a small sample of capillary blood with a lancet, either from the fingertip or forearm. Optimal metabolic control is possible for these patients with a minimum of four blood glucose checks per day.\(^3\) However, for children and adolescents with Type 1 diabetes undergoing insulin therapy, this method of glucose monitoring is often recommended up to eight times daily.\(^4\) The resulting discomfort limits patient compliance and leads to suboptimal blood glucose control as well as the risk of hypoglycemic incidents. The development of a pain-free, non-invasive sensing technique would greatly improve patient compliance and, ultimately, patient outcomes. At the same time, the ideal technology is to continuously monitor glucose levels in blood with an implantable sensor. This thesis focuses on developing/evaluating new approaches/devices that contribute to both goals. A major portion of this chapter was published as a review paper in *Applied Materials Today*.\(^5\)
1.2 Non-Invasive Glucose Monitoring Systems

Many studies have aimed to establish a useful yet noninvasive technique for monitoring blood glucose levels. The methods applied include infrared (IR) spectroscopy, Raman spectroscopy, optical coherence tomography, measurement of tissue metabolic heat conformation and electrochemical analysis of sweat, however, none of these methods have proven to be clinically applicable. In addition, several in vivo sensing approaches have been proposed, including fluorescence spectroscopy, surface plasmon resonance of nanoparticles, electrical impedance measurements, and implantable/subcutaneous amperometric glucose sensors. Among these, only subcutaneous electrochemical glucose sensors are available commercially, although frequent calibration, short in vivo lifetimes, limited accuracy, and high cost have limited their widespread use. Further, such sensors are clearly much more invasive.

Measurement of tear glucose concentrations is an emerging area of research and several studies have reported a possible clinically useful correlation between blood and tear glucose levels. Beca et al. reviewed studies of this correlation using different detection methods, concluding that there is evidence of a correlation. Methods for tear glucose measurement require a low micromolar (µM) range for the limit of quantitation (LOQ), high selectivity over potential interferences (e.g., ascorbic acid and uric acid), and measurement within µL sample volumes. To date, tear glucose has been measured by capillary electrophoresis (CE) coupled with laser-induced fluorescence (LIF), fluorescence sensors, liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (ESI-MS), holographic glucose sensors, a miniaturized flexible thick-film flow-cell electrochemical detector, a strip-type flexible biosensor and an amperometric biosensor with microflow injection analysis. Badugu et al. and Ruan et al. also
reported the feasibility of using disposable contact lenses to monitor tear glucose.\textsuperscript{31-33} A study from the Asher laboratory\textsuperscript{26} employed a LC-ESI-MS technique to measure glucose concentrations in 1 \textmu L tear samples obtained from non-diabetic subjects and reported a median value of 28 \textmu M with a range from 7-161 \textmu M for this group.

1.3 Nitric Oxide Release for Improving Performance of More Invasive Implantable Chemical Sensors

1.3.1 Challenge

The real-time and accurate measurements of clinically important species, such as arterial blood gases (pH, $PO_2$, $PCO_2$), electrolytes ($Na^+$, $K^+$, $Ca^{2+}$) and glucose/lactate levels in undiluted circulating whole blood of hospitalized patients via implantable chemical sensors continues to be of great interest to improve the quality of health care.\textsuperscript{34-36} This is because intermittent \textit{in vitro} blood measurements cannot provide real-time information on changing physiological conditions of critically ill patients, which can lead to life-threatening events. However, designing successful \textit{in vivo} sensors with sufficient accuracy for clinical use is complicated by adverse biological responses that lead to errant analytical results.\textsuperscript{37-39}

The main biological response to implanted sensors depends largely on the location of implantation: blood or subcutaneous tissue.\textsuperscript{40} When an implantable chemical sensor is in contact with flowing whole blood, the initial biological response is the nearly instant adsorption of plasma proteins (e.g., fibrinogen, fibronectin, vitronectin and von Willebrand's factor (vWF)) onto the foreign device surface, which leads to the adhesion, activation and aggregation of platelets that trap red blood cells and ultimately forms a thrombus (blood clot) within a matter of hours (see Fig. 1.1).\textsuperscript{41-44} Such biofouling on the surface of implantable sensors can restrict the mass transport of analytes to the
sensing surface, leading to deterioration in sensor response/performance.\textsuperscript{45-46} Further, the metabolic activities of adhered cells consume oxygen and glucose while producing carbon dioxide, thereby altering the local concentration of these species measured by the implanted chemical sensors, leading to inaccurate results.\textsuperscript{39}

Sensors implanted in the subcutaneous tissue (under the skin) are also subject to biocompatibility problems, primarily from inflammatory responses by the body's self-defense mechanism which can result in inaccurate results.\textsuperscript{37,46} This acute inflammatory response rapidly takes place after sensor implantation as proteins and inflammatory cells migrate to the implant site and adhere to
the sensor surface. Subsequently, local edema is created at the surface. Further, phagocytic cells (e.g., neutrophils, monocytes, and macrophages) form fibrous encapsulation around the sensors, creating a significant change of the analyte diffusion rate, resulting in frequent changes in the response curve/sensitivity of the sensor. In addition, the injury to blood vessels caused by implanting chemical sensors in the subcutaneous tissue will facilitate the diffusion of the analyte from the blood to the subcutaneous fluids affecting the output accuracy of sensors. To date, attempts to monitor in subcutaneous tissue have mostly been focused on glucose measurements, since the concentrations of glucose in this tissue closely relate to blood levels. The critical parameters for animal models and data processing methods required to assess the reliability and usefulness of such subcutaneous glucose sensors have also been studied.

Many strategies have been reported to reduce biofouling on the surface of implanted chemical sensors by using various types of modified polymers. Some examples include hydrogels, phospholipid-based biomimicry, flow-based systems, Nafion polymer coatings, surfactant-derived membranes, diamond-like carbons, use of polyurethane and silicone elastomers, and topology treatments. Also, many efforts have been made to design “active” biocompatible materials, most often by immobilization of heparin, to prevent significant thrombus formation at the surface of intravascular devices. However, platelet cell adhesion and activation can still occur, leading to localized changes in analyte levels. Furthermore, the use of immobilized heparin may be problematic for patients who suffer from thrombocytopenia, hemophilia or renal failure. Despite such extensive efforts, none of these methods have proven to be useful for fabrication of clinically effective implantable chemical sensors for use in monitoring critically ill hospital patients.
1.3.2 Nitric Oxide

Nitric oxide (NO) is a diatomic free radical and endogenously produced from L-arginine and oxygen via nitric oxide synthase (NOS) enzyme within the endothelial cells that line the inner walls of all blood vessels.\(^7\) Nitric oxide is known to prevent platelet activation/adhesion and subsequent thrombosis, as well as serving as an inhibitor of bacterial cell proliferation and biofilm formation.\(^73\)-\(^76\) Indeed, macrophage cells in our bodies, which fight infection, produce high levels of NO for this purpose. Further, it is known that NO at low levels is also a potent anti-inflammatory agent.\(^77\) Hence, the prevention of thrombosis, infection, and foreign body response associated with implantable chemical sensors may be possible by incorporating them within platforms capable of releasing low levels of NO at physiological fluxes of ca. 0.5–4.0 \(\times 10^{-10}\) mol·cm\(^{-2}\)·min\(^{-1}\).\(^78\) Nitric oxide concentrations can be most accurately measured via a chemiluminescence method.\(^79\)

It is also widely known that NO can be highly reactive with \textit{in vivo} species (oxyhemoglobin, oxygen, and thiols). Indeed, the life-time of NO in blood is very short, <1 s.\(^43\) Nonetheless, its lifetime is still long enough to have important physiological activities at contact surfaces, especially serving to inhibit platelet activation (preventing clotting when produced at flux rates that mimic those produced by endothelial cells). NO may also be considered as a potential interference for \textit{in vivo} oxygen sensing. However, Ren et al. showed that an implantable amperometric O\(_2\) sensor is fully compatible with NO release, as no noticeable amperometric signal changes were observed for the O\(_2\) sensor with NO generation at physiological flux levels.\(^80\) Further, Nichols et al. evaluated the \textit{in vivo} glucose recovery of subcutaneously implanted NO-releasing (from saturated NO solution) microdialysis probes in a rat model over 14 d period and reported that intermittent sustained NO release from implanted probe surfaces may improve
glucose diffusion for such subcutaneously implanted devices by mitigating the foreign body reaction.\textsuperscript{81} However, since NO has a short half-life, many NO donors have been studied for achieving prolonged and controlled NO delivery for potential clinical use, instead of using NO gas from a cylinder.

1.3.3 NO-releasing Chemical Sensors

1.3.3.1 N-Diazeniumdiolates as NO donors

A N-diazeniumdiolate is an NO adduct with secondary amines,\textsuperscript{82} and one of the most widely investigated NO donors due to its ability to release NO spontaneously under physiological conditions via a proton-driven reaction.\textsuperscript{83-84} One equivalent of amine reacts with two equivalents of NO to form the corresponding diazeniumdiolate under high NO(g) pressure (e.g., 80 psi). An initial study by Espadas-Torre et al. reported the fabrication of classical polymer membrane type ion-selective electrodes (ISE) for H\textsuperscript{+} and K\textsuperscript{+} with a NO donor (N, N\textsuperscript{′}-dimethylhexanediamine nitric oxide adduct, also known as DMHD/N\textsubscript{2}O\textsubscript{2}) within to investigate whether low level of continuous NO release from the diazeniumdiolate species would impair the analytical performance of the sensors.\textsuperscript{85} The results showed that ISE sensors with and without DMHD/N\textsubscript{2}O\textsubscript{2} exhibited very comparable potentiometric responses: K\textsuperscript{+}-sensors with and without the added diazeniumdiolate species (membrane composition: 66% Tecoflex polyurethane/30% dioctyl sebacate/0.6% potassium tertakis(4-chlorophenyl)borate/2.5% valinomycin) exhibited slopes of 60.3 ± 0.1 and 60.6 ± 0.2 mV/decade and intercepts of −5.31 and −5.44, respectively. For H\textsuperscript{+}-detection (membrane composition: 33% Tecoflex polyurethane/63% dioctyl sebacate/1.7% potassium tertakis(4-chlorophenyl)borate/2.5% tridodecylamine) the sensors exhibited slopes of 58.5 ± 0.9 and 59.7 ± 0.6 mV/decade with intercepts of −9.38 and −9.50, respectively. This study also
reported that the surfaces of the sensors exhibit a marked decrease in thrombogenic properties when measured by *in vitro* platelet adhesion studies using sheep plasma. This early study clearly suggested that their approach for enhancing biocompatibility of ion sensors by incorporation of a NO donor can be applicable to a wide range of polymeric materials that can be used in implantable chemical devices. In another early study, Mowery et al. prepared Clark style amperometric oxygen sensors and potentiometric sensors (pH and K⁺) with three distinctly different types of diazeniumdiolate NO donors, DMHD/N₂O₂, linear polyethylenimine/N₂O₂ (LPEI/N₂O₂) and methoxymethyl piperazine PVC/N₂O₂ (mompipPVC/N₂O₂), and reported a significant decrease in platelet adhesion and activation for both *in vitro* and *in vivo* sensors fabricated with these materials.86

The effect of diazeniumdiolate species was further investigated for potential improvement of the biocompatibility and *in vivo* analytical performance of intravascular amperometric oxygen sensors by Schoenfisch et al. In this work, the authors fabricated NO-releasing silicone rubber-catheter-based amperometric PO₂ sensors via dip-coating the sensor with gas permeable coatings formulated with cross-linked silicone rubber containing DMHD/N₂O₂. They then demonstrated that there was greatly reduced thrombus formation on the outer surface of the sensors and superior overall sensor performance vs. control sensors when implanted, in the absence of systemic anticoagulation, within the carotid and femoral arteries of mongrel dogs for 6-24 h.87 The only caveat was leaching of N,N′-dimethyl-hexane-diamine and the corresponding toxic nitrosamine, a decomposition product of DMHD/N₂O₂, into aqueous soaking solutions or blood. Therefore, they concluded that NO donors such as diazeniumdiolates that possess a more lipophilic structure, or can be covalently attached to the outer polymer coating of the sensor would be more optimal.
Frost et al. decreased the potential problem of leaching diamine and nitrosoamine by using a more lipophilic NO-releasing agent within the silicone rubber polymer that contains a diazeniumdiolate species (DACA-6/N$_2$O$_2$), anchored to the cross-link sites of a silicone rubber matrix.\textsuperscript{88} They further prepared Clarke-style amperometric oxygen-sensing catheters coated with an outer layer of the DACA-6/N$_2$O$_2$ polymer that releases physiological levels of NO for $>20$ h, and showed significantly improved biocompatibility and analytical performances compared to control sensors without NO release when tested \textit{in vivo} within the carotid and femoral arteries of swine for 16 h.

By coating a dual lumen silicone catheter with this DACA-6/N$_2$O$_2$-silicone layer, an NO release potentiometric CO$_2$ sensor was also developed.\textsuperscript{89-90} The wall between two lumens was doped with a proton ionophore, tridodecylamine, and a tetraphenylborate type cation exchanger, functioning as a pH-selective layer. One lumen contained a bicarbonate/sodium chloride solution and a Ag/AgCl electrode. The pH of this lumen solution decreased in the presence of an increase in the $P_{CO_2}$ level in solution (or blood) surrounding the device. This causes a potential change across the polymeric wall between the lumen filled with the bicarbonate solution and the other lumen filled with strong buffer (4-morpholinoethanesulfonic acid buffer, pH = 5.7). A second Ag/AgCl reference electrode in the buffered lumen provides a means to measure this potential change as a function of change in $P_{CO_2}$ levels. The overall potential change of this two-electrode system was found to be proportional to log($P_{CO_2}$) of the sample with a near-Nernstian slope ($-60.1$ to $-57.8$ mV/decade) even in the presence of $\sim 10 \times 10^{-10}$ mol·cm$^{-2}$·min$^{-1}$ fluxes of NO from the surface of the device.
Given that the NO-releasing implantable PO₂ sensors showed promising in vivo results, Gifford et al. developed the very first NO-releasing needle-type enzyme-based electrochemical glucose sensors for measurement of glucose in subcutaneous fluid. The outer membrane of this glucose sensor contained a diazeniumdiolate species, (Z)-1-[N-butyl-N-[6-(N-methylammoniohexyl)amino]]-diazen-1-ium-1,2-diolate (DBHD/N₂O₂; see Fig. 1.2 for chemical structure), that can release NO for ~16 h. The resulting sensor showed excellent analytical performance for both in vitro and in vivo tests and inhibited the typical subcutaneous inflammatory response when implanted in Sprague-Dawley rats for 3 d. Clarke error grid analysis indicated superior performance for the NO-releasing sensors when compared to controls on both days 1 and 3. Histological examination of the sites where the NO-releasing sensors were placed in the animals also showed significantly less inflammatory response over the first 24 h compared to non-NO

Figure 1.2. The structures of some NO donors/generators: (a) (Z)-1-[N-butyl-N-[6-(N-methylammoniohexyl)amino]]-diazen-1-ium-1,2-diolate (DBHD/N₂O₂), (b) S-nitroso-N-acetyl-D-penicillamine (SNAP), and (c) Copper(II)-tri(2-pyridylmethyl)amine (Cu(II)TPMA), a catalyst used for electrochemical generation of NO from an inorganic nitrite reservoir.
release controls, and a significant decrease in the \textit{in vivo} sensor signal stabilization time right after implantation. Yan et al. and Wolf et al. also developed the same type of implantable amperometric glucose sensors based on hydrogen peroxide detection with an outer polymer containing NO releasing DBHD/N\textsubscript{2}O\textsubscript{2} and found reduced thrombus formation and improved analytical

\textbf{Figure 1.3.} (a) NO-releasing needle/catheter type glucose sensor with DBHD/N\textsubscript{2}O\textsubscript{2} as NO donor; (b) photos of the control (top) and NO release (bottom) glucose sensors after the \textit{in vivo} experiment. The portions of the sensors to the left of the dashed lines were actually located inside the veins; (c) Comparison of glucose concentration values obtained from benchtop blood gas analyzer and the converted current values measured by the continuous sensor. One conversion of current to glucose concentration (mmol·L\textsuperscript{-1}) was made with the calibration curve in bovine serum; the other conversion was a one-point calibration. Adapted from Ref. 93.
performance compared to control sensors when implanted in the veins of rabbits for 7 h (see Fig. 1.3). However, these studies found that NO release from a thin layer of DBHD/N₂O₂ was too short and uncontrollable for ultimate clinical application and that NO could also be detected as an interference current signal at an applied potential of +0.6 V vs. Ag/AgCl and higher on the underlying platinum–iridium working electrode, which was required for electrochemical glucose sensor measurement via detection of hydrogen peroxide produced from glucose oxidase. Overall, these studies indicated that NO is compatible with subcutaneously and intravascularly-implanted electrochemical glucose sensors, but additional work on the best NO release chemistry is still necessary.

The need for improved NO release coatings to enhance the in vivo performance of implantable chemical sensors led to the development of an NO-releasing hydrophilic platform. Marxer et al. first developed an amperometric sol-gel derived oxygen sensor that releases NO via coating the platinum working electrode with an aminosilane/ethyltrimethoxysilane hybrid xerogel film doped with hydrophilic polyurethane (HPU) that are subsequently exposed to 5 atm of NO for 3 d (to form diazeniumdiolate groups on the amine sites). The sensors exhibited highly reproducible and linear sensitivities to oxygen with rapid response times and released NO at fluxes of 7.2 × 10⁻¹⁰ mol·cm⁻²·min⁻¹, effectively reducing platelet adhesion for 48 h. However, it was suggested that further work to improve the NO release layer was needed to evaluate the in vivo performance of such implantable chemical sensors. Indeed, Shin et al. developed a macro glucose sensor based on a new type of hybrid sol-gel/polyurethane (PU), made by sequentially coating the platinum working electrode with the following four polymeric membranes: a sol-gel layer with immobilized glucose oxidase (GOX), PU for protection of the GOX, PU doped with NO donor
(diazeniumdiolated sol-gel particles) and sol-gel particles, and then PU as an outermost layer. The NO donor was prepared by exposing aminosilane-based sol-gel particles to 5 atm NO for 3 d to convert accessible amine groups to diazeniumdiolates. The design demonstrated good sensitivity, reproducibility, and fast response time to glucose as well as a reduction of enzyme inactivation. However, the authors observed leaching of sol-gel particles even when the sensor was coated with an outermost PU layer and a drastic decrease in sensitivity of sensor to glucose when coupled with this new NO-releasing sol-gel film. Also, a longer duration of higher NO release fluxes was still required for the miniaturized version of sensor to study the in vivo biocompatibility of such a design. Indeed, Oh et al. reported a miniaturized glucose biosensor modified with a NO-releasing xerogel micro-patterned array that released NO from 5 µm wide lines separated by 5 or 20 µm distances. The micro-patterned array allowed for an increase in glucose sensitivity since significant portions of the sensor’s surface remained unmodified by the NO release coating. They found that sufficient levels of NO generation from such a design significantly reduced platelet adhesion (>40% reduction) and Pseudomonas aeruginosa bacterial growth (70-80% reduction) without reducing enzymatic activity of GOX. However, since the NO release from sensor depleted after only 3 days, extension of NO release time would be necessary for longer-term in vivo testing of such miniaturized glucose biosensors.

Koh et al. further investigated the fabrication of polyurethane-based implantable glucose sensors with a NO-releasing porous fiber mat-modified sensor membrane (540 ± 139 nm fiber diameter, 94.1 ± 3.7% porosity). The sensor released ~100 nmol of NO per mg of polyurethane over 6 h period without leaching of the NO donor, even in serum. However, to date, this new design has never been evaluated for in vivo analytical performance.
A diazeniumdiolate type-NO donor was also used to provide NO release for film type fluorescent oxygen sensors. In initial efforts, the fluorescent oxygen indicator (pyrene/perylene donor/acceptor pair) was doped into SR membrane with dispersed DMHD–N₂O₂ particles or DACA-6-modified SR membrane. However, the oxygen response became non-linear because of heterogeneity of the particle-doped film and/or a fluorescence quenching effect from the amine groups of the NO donor. This problem was solved by a two-layer configuration on a quartz slide, which consisted of an underlying DACA-6 modified SR membrane for NO release and an outer SR membrane doped with the oxygen indicator for fluorescence-based oxygen sensing. By using this configuration, only NO gas rather than the NO donor interacted with the oxygen indicator and the linearity and sensitivity toward oxygen was not perturbed. NO release at a flux of >4 × 10⁻¹⁰ mol·cm⁻²·min⁻¹ was obtained for at least 24 h at room temperature and the sensor response was quite stable during this period.

Obviously, the quartz slide-based sensor is not ideal for in vivo use. Rather, optical fiber sensor technology has been used successfully for in vivo diagnostic applications. Therefore, the two-layer configuration was expanded to an optical fiber type sensor configuration. Dobmeier et al. developed a NO-releasing fiber optical pH sensor. An aminoalkoxysilane-based xerogel membrane with covalently bonded N-diazeniumdiolates as the NO donor was deposited on the tip of optical fibers. Then the same type of xerogel doped with fluorescent pH indicator, seminaphthorhodamine-1 carboxylate, was coated on the top of the NO donor layer. NO release at >0.24 × 10⁻¹⁰ mol·cm⁻²·min⁻¹ flux was obtained for up to 16 h, indicating the good permeability of NO through the pH-sensitive coating. The underlying NO-releasing layer allows transmission
of excitation and emission light and does not interfere with the fluorescent pH detection. Response to physiologically relevant pH values (7.0-7.8) were obtained by this sensor with a short response time, good linearity, and high precision. Also, in vitro platelet adhesion studies confirmed the effectiveness of NO released from such sensor device to inhibit of adhesion of platelets from porcine serum samples.

1.3.3.2 Nitrite for NO generation

To avoid the leaching of NO donors and/or byproducts into the bloodstream, and to produce very controllable NO generation over prolonged time, Ren et al. reported improvement in in vivo analytical performance of intravascular analytical sensors by coupling them with a new electrochemical method of NO generation. This method involves electrochemical reduction of inorganic nitrite ions catalyzed by a copper(II)-ligand complex, copper(II)-tri(2-pyridylmethyl)amine (Cu(II)TPMA; see structure in Fig. 1.2). A dual lumen catheter-type amperometric PO2 sensor design was developed (with electrochemical NO generation in the bigger lumen and PO2-sensing platinum–iridium electrode in smaller lumen; see Fig. 1.4) and these devices were implanted in both veins and arteries of rabbits and pigs for up to 21 h. The NO generating sensors exhibited less clot formation and much more accurate analytical results as compared to controls. The advantage of this sensor design is that the amount NO generation can be easily controlled by different applied potentials of between −0.2 and −0.4 V vs. Ag/AgCl to the working electrode of the NO generating lumen. Further, the NO release can then be toggled “on” and “off” by disconnecting the electrode leads from that lumen to the potentiostat. Furthermore, the duration of NO generation can be readily extended by increasing the volume or concentration of nitrite within the reservoir. Overall, the study demonstrated that this method could significantly improve
the biocompatibility of a wide variety of implantable chemical sensors and could provide a potential tool for longer-term in vivo investigations into the interplay between long-term NO release and extended in vivo analytical performance.

1.3.3.3 S-Nitrosothiols (RSNOs) as NO donors

S-Nitrosothiols (RSNO) are another class of NO donors that can be utilized to improve the biocompatibility of implantable chemical sensors. They are transporters and/or storage vehicles of NO in the body and can be rapidly decomposed to release NO via a several different pathways, including thermal and photolytic cleavage of the S-N bond of the RSNO to release NO. In addition, Cu$^+$ species (formed from reduction of Cu$^{2+}$) can catalyze the reaction of RSNOs to liberate NO. Furthermore, ascorbate-mediated reactions can occur in which ascorbate at low

Figure 1.4. (a) Schematic of dual-lumen catheter-type electrochemical NO generating/releasing $PO_2$ sensor with cross-section geometry of catheter; (b) representative photo illustrating the degree of clot formation on the surface of the control and the NO releasing sensors implanted in rabbit veins for 7 h; (c) average thrombus coverage percentage on NO releasing sensors vs. control sensors. (n = 5 rabbits, p < 0.05); (d) representative current response for a NO releasing sensor (black) and a control sensor (red) (implanted in pig arteries for 21 h) compared with blood draw in vitro test values (reference method, blue squares). Arrows indicate where fraction of inspired oxygen (FiO$_2$) changes from 21% to 100%. Figure adapted from Ref. 100.
concentration can reduce trace copper ion impurities in solution (i.e., Cu$^{2+}$) to generate Cu$^{+}$ that can subsequently react with RSNOs to produce NO.

Soto et al. were the first to develop implantable chemical sensors with an RSNO as the NO donor source and reported the first long-term investigation on the *in vivo* analytical performance of percutaneously implanted NO-releasing amperometric glucose sensors in swine models. The sensors were implanted for 10 d and analytical results were obtained comparing the NO-releasing sensors with two different NO-releasing rates with the same total amounts of NO released (3.1 µmol·cm$^{-2}$): rapid NO release (16.0 ± 4.4 h) from N-diazeniumdiolate NO donor (MAP3/NO) and slower NO release (>74.6 ± 16.6 h) from S-nitrosothiol-modified silica nanoparticles (MPTMS-RSNO). It was shown that the sensors with slower NO-release rate for extended durations exhibited a significantly lower mean absolute relative deviation (MARD) on days 1 and 3 (26.0 ± 5.1 and 23.9 ± 8.6%, respectively) versus controls (34.3 ± 10.9 and 38.8 ± 10.4%, respectively), and were characterized by shorter sensor lag time (<4.2 min) in response to intravenous glucose tolerance tests when compared to rapid NO-releasing and controls sensors (>5.8 min) at 3, 7, and 10 d. Overall, both rapid and slower NO-release sensors exhibited improved accuracy vs. controls and it was suggested that materials that are capable of releasing a large amount of NO with even longer duration (i.e., several weeks) would be necessary to create the ultimate NO-release strategy for long-term implantable chemical sensing technology. Indeed, Soto et al. further investigated fabrication of an implantable glucose sensor using an optimized HP-93A-100 PU membrane doped with RSNO-modified silica nanoparticles as the outer, glucose diffusion-limiting layer. The resulting sensor yielded a linear glucose calibration between 1 and 21 mM over a 2-week period while incubating in PBS. Further, the sensing device released NO.
at >0.8 pmol·cm$^{-2}$·s$^{-1}$ for up to 6 d with negligible particle leaching (<0.6%). However, *in vivo* analytical performance of this new design has not yet been reported in the literature.

Of many RSNOs available for use in developing NO release implantable sensors, SNAP (*S*-nitroso-*N*-acetyl-penicillamine; see chemical structure shown in Fig. 1.2, above) has been intensively investigated to prevent donor leaching and stabilize release of NO for improvement in the biocompatibility of various implantable medical devices, including intravascular chemical sensors. Cha et al. (Chapter 5 in this thesis) demonstrated the compatibility of NO with implantable enzymatic amperometric glucose sensors based on the osmium (III/II) mediated electrochemistry and showed that amperometric glucose response is preserved in the presence of continuous NO release from the surrounding SNAP-doped silicone tubing (see Fig. 1.5) when tested in both phosphate buffer (pH 7.4) and *in vitro* within heparinized whole porcine blood at 37 $^\circ$C (see Chapter 5 for more details).$^{109}$

1.3.3.4 Endogenous RSNOs for NO generation

One common limitation of various NO release systems is the finite amount of NO donors that can be held within the sensor device. The NO donor will be depleted typically within a time duration of one day to one month and the biocompatibility benefit will no longer exist after the termination of NO release. To overcome this issue, generation of NO at the surface of biomedical implants from endogenous NO donors such as *S*-nitroso-albumin, *S*-nitroso-glutathione, *S*-nitroso-cysteine, and nitrite has been suggested. Duan and Lewis modified biomedical polymers with L-cysteine and found that such surfaces can generate NO from *S*-nitroso-albumin via a NO transfer and release mechanism.$^{110}$ However, the decomposition of nitrosated cysteine may convert L-cysteine
to its disulfide dimer product that is unable to generate NO. Other researchers have examined a series of metal ion-based catalysts embedded within or tethered to a polymer coating of biomedical implants for in situ generation of NO from endogenous RSNOs.\textsuperscript{111-117} For example, copper complexes, organoselenium species, organoditelluride species, and copper particles on the implants have been found to catalyze decomposition of various endogenous NO donors in the presence of reductants such as ascorbic acid or glutathione.

Wu et al. adapted the concept of NO generation to intravascular chemical sensors. In this work, 3 μm and 80 nm Cu\textsuperscript{0} particles were doped into a polyurethane or polyurethane/silicone rubber layer coated on a silicone rubber catheter type amperometric oxygen sensor.\textsuperscript{117} Slow corrosion of the
Cu\(^0\) particles generates copper ions, which catalyzes decomposition of GSNO (a naturally present RSNO in blood) in the presence of GSH (also present in blood). The NO-generating oxygen sensors were implanted into porcine arteries for 19–20 h. A lower degree of thrombus formation and an enhanced oxygen sensing accuracy were observed on such sensors compared to control sensors fabricated without the Cu\(^0\) particles coating. However, it was found that the effectiveness of this NO generation method can vary between animal species/subjects. Some animals show significant clots on the Cu\(^0\) particle-modified catheters, suggesting that these animals may have a lower amount of circulating endogenous RSNOs (probably GSNO) in blood stream and are unable to generate high enough NO levels at the surface of implantable sensors to prevent thrombus formation.

1.4 Summary

The prospect of developing NO-releasing or generating real-time continuous monitoring sensors that respond to clinically important ions, gases and molecules in blood or subcutaneous fluid is an ambitious effort to solve a very challenging analytical problem. A variety of NO release/generation strategies have been shown to release or generate sufficient amounts of NO to enhance the biocompatibility and \textit{in vivo} analytical performance of both intravascular and subcutaneous chemical sensors. However, additional efforts are still needed to devise a platform that can achieve stable release or sufficient generation NO levels for 1-2 weeks and also be compatible with the several different types of sensing chemistries and sensor designs required for continuously monitoring across the spectrum of the clinically important blood analytes. If such sensors can ultimately be realized through continued research efforts, then commercial
development of in vivo for routine in vivo monitoring would benefit patients in intensive care units around the world.

1.5 Statement of Research

As previously described in the chapter, many efforts have contributed to the development of non-invasive and continuous glucose monitoring systems. Recent studies of non-invasive glucose monitoring systems utilizing glucose in tear fluids have been limited by their ability to achieve the low micromolar range LOQ and high selectivity required using a microliter volume of tear fluid sample. Therefore, the first goal of this thesis is to employ a new method involving blood glucose test strips, to investigate whether any of these strips can achieve the LOQ values required for tear glucose measurements. As reported in Chapter 2, the Roche Accu-Chek type test strips were found to have very low LOQ values and can potentially be applied for human studies.

The second part of this dissertation will focus on investigating the compatibility of nitric oxide release with implantable/continuous osmium(III/II)-mediated glucose sensors. Since SNAP was found to be exceptionally stable when doped into low water uptake biomedical polymers, such as silicone, SNAP is chosen as the nitric oxide source to be coupled with the osmium-mediated glucose sensor for potential application as intravascular/continuous glucose sensors. This dissertation includes in vitro and in vivo studies using such sensors to further investigate the validity of the design of NO-releasing osmium-mediated glucose sensor.

Overall, this chapter (Chapter 1) has summarized and reviewed, in detail, the research efforts to date regarding the development of non-invasive and implantable/continuous glucose monitoring


Chapter 3 focuses on an investigation into the origin of the very low detection limit and high selectivity toward glucose exhibited by Roche Accu-Chek test strips that enables measurement of tear glucose levels. The conclusions of this study suggest that the unique combination of a nitrosoaniline derivative and a recombinant PQQ-dependent glucose dehydrogenase are the key elements that make the Roche strip so selective and sensitive, enabling direct tear glucose measurements with such strips. This work was published in *Electroanalysis* (K. H. Cha, Y. Qin, M. E. Meyerhoff, *Electroanalysis* 2015, 27(3), 670-676).

Chapter 4 provides data from a further investigation into the potential correlation between tear and blood glucose levels in a small clinical study involving diabetic human subjects and reports a moderate correlation between tear and blood glucose in most of the patients tested. However, the study results also suggest that a longer-term analysis of individual patients (beyond the 1.5 h test period used in this study) may be key to definitively establish the correlation between tear and blood glucose levels.
Chapter 5 focuses on studying compatibility of nitric oxide release from SNAP with implantable enzymatic sensors based on osmium (III/II) mediated electrochemistry and reports that such sensors maintain their functionality, sensitivity and accuracy for detecting glucose levels in blood in the presence of NO release that is high enough to prevent clotting for at least 3 days. This work was published in *ACS Sensors* (K. H. Cha, M. E. Meyerhoff, *ACS Sensors* 2017, 2(9), 1262-1266).

Chapter 6 summarizes the major findings/conclusions from this dissertation research and provides suggestions for future directions that will build upon the studies reported herein.

1.6 References

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CHAPTER 2

Evaluation of Commercial Glucometer Test Strips for Potential Measurement of Glucose in Tears


2.1 Introduction

The World Health Organization (WHO) reports that more than 347 million people worldwide live with diabetes. Over 1.1 million deaths in 2005 were caused by diabetes, and deaths attributed to diabetes are estimated to double by 2030.1 Regular measurement and control of blood glucose is essential to avoid life-threatening hyper- and hypoglycemic events and associated serious long-term complications.2 Normally, a personal blood glucometer with a test strip allows diabetics to monitor their blood glucose level by obtaining a small sample of capillary blood with a lancet, either from the fingertip or forearm. Optimal metabolic control is possible for these patients with

* The principal experiments of this chapter were conducted by Kyoung Ha Cha, with the help of Dr. Gary C. Jensen in understanding the design of glucometer test strips, Anant Balijepalli in helping perform the clinical study, and Dr. Bruce E. Cohan in funding the entire study.
a minimum of four checks per day. However, for children and adolescents with Type 1 diabetes on insulin therapy, this method of glucose monitoring is often recommended up to eight times a day. The resulting discomfort can limit patient compliance and lead to suboptimal blood glucose control as well as the risk of hypoglycemia. The development of a pain-free, non-invasive sensing technique would greatly improve patient compliance and, ultimately, patient outcomes.

Many studies have aimed to establish a useful yet noninvasive technique for blood glucose level monitoring. The methods applied include infrared (IR) spectroscopy, Raman spectroscopy, optical coherence tomography, and measurement of tissue metabolic heat conformation; however, none are currently proven to be clinically applicable. In addition, several in vivo sensing approaches have been proposed, including fluorescence spectroscopy, surface plasmon resonance of nanoparticles, electrical impedance measurements, and implantable/subcutaneous amperometric glucose sensors. Among these, only subcutaneous electrochemical glucose sensors are available commercially, although frequent calibration, short in vivo lifetimes, limited accuracy, and high cost have prevented their widespread use.

Measurement of tear glucose concentrations is an emergent research focus, and several studies have reported a possibly clinically relevant correlation between blood and tear glucose levels. Beca et al. recently reviewed studies of this correlation through different detection methods, concluding that there is evidence of a correlation; however, further studies are needed to determine the potential utility of tear glucose measurements to help achieve tight, yet safe, glycemic control.
Methods for tear glucose measurement mandate a low micromolar range LOQ, high selectivity over potential interferences (e.g., ascorbic acid and uric acid), and measurement within µL sample volumes. Tear glucose has been measured by capillary electrophoresis (CE) coupled with laser-induced fluorescence (LIF),\textsuperscript{21} fluorescence sensors,\textsuperscript{22} liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (ESI-MS),\textsuperscript{23} holographic glucose sensors,\textsuperscript{24} a miniaturized flexible thick-film flow-cell electrochemical detector,\textsuperscript{25} and a strip-type flexible biosensor.\textsuperscript{26} Badugu et al. also reviewed the feasibility of using disposable contact lenses to monitor tear glucose.\textsuperscript{27-28} A recent study from the Asher laboratory\textsuperscript{23} employed a LC-ESI-MS technique to measure glucose concentrations in 1 µL tear samples obtained from non-diabetic subjects and reported a median value of 28 µM with a range from 7 - 161 µM for this group. For a comprehensive review of electrochemical glucose sensors, including applications related to tear glucose, readers may reference Wang et al.\textsuperscript{29}

Electrochemical measurement of blood glucose with enzyme-based glucometer test strips is the standard method for diabetic near-patient and self-patient care and may be applicable to measure tear glucose concentration. Recently, a sensitive electrochemical technique for measurement of tear glucose, utilizing needle-type amperometric glucose sensors inserted into small glass capillary tubes, was applied to tear samples from anesthetized rabbits.\textsuperscript{17} This method provided comparable detection limits to the LC-ESI-MS technique with reduced time, effort, and cost investment. Results showed a clear correlation between blood glucose and tear glucose levels, but the ratio varied from between individual animal subjects, perhaps due to differing tear production rates. However, the electrochemical sensors employed in these studies required 3 - 5 µL sample volumes, which is too large in quantity for application in studies involving human subjects.
Herein, we examine the use of commercial electrochemical blood glucose glucometer strips requiring $< 1 \mu L$ to measure glucose in tears. While these strips are designed to measure blood glucose in the range of 3 - 20 mM, testing strips from several manufacturers with a laboratory potentiostat to control the applied potential and measure low currents showed that strips employing glucose dehydrogenase (GDH) as the active enzyme yield output currents that provide reproducible and linear amperometric responses to glucose in the 0 - 100 $\mu$M range. Specifically, strips manufactured by Roche Diagnostics which utilize pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) as the active enzyme and a nitrosoaniline derivative as the electron transfer mediator yield the best analytical performance. Further, optimizing the potential applied to the working electrode in these strips results in relatively low interference from both ascorbic and uric acid. It will be shown that measurements of glucose with these strips using tear samples from normal human subjects were found to be in the range reported by Asher’s group, suggesting that these strips could provide a simple tool for more extensive clinical studies to determine whether tear glucose sufficiently correlates with blood to be useful as a supplementary method to manage diabetic patients.

2.2 Experimental Section

2.2.1 Chemicals and Reagents

D-(+)-glucose, sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na$_2$HPO$_4$), potassium phosphate monobasic (KH$_2$PO$_4$), L-ascorbic acid, uric acid, and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO).
2.2.2 Sensitivity and Selectivity Analysis

To determine the sensitivity and selectivity of commercial glucometer strips, a four-channel Biostat (ESA Biosciences Inc., Chelmsford, MA) measured the amperometric response to glucose solutions (0 - 100 µM) and to glucose solutions (0 - 100 µM) containing potential interfering species (easily oxidizable species at applied potentials). For testing of the commercial glucose strips, the reported applied potentials are versus the internal reference/counter electrode within the strips. The measured currents reported are a ten second average value from an indicated starting measurement time-point following sample introduction. A 5 mM stock solution of glucose was made in phosphate saline buffer (PBS) solution containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 2.0 mM KH$_2$PO$_4$, adjusted pH to 7.4 with 1.0 M NaOH. Standard solutions were prepared by dilution of this stock solution using PBS solution for calibrations of glucose at 0, 50, and 100 µM. More detailed assessment of low-end analytical performance for the human study employed calibration curves for glucose standards at 10, 50 and 100 µM. Glucose solutions of 25, 50, 75, and 100 µM containing 10 µM acetaminophen, 100 µM ascorbic acid (freshly made each day), and 100 µM uric acid were used to examine the cumulative effect of these potential interference species on the accuracy of glucose measurements. The reported average levels of ascorbic and uric acid in tears are 20 ± 6.2 and 68 ± 46 µM,$^{31,32}$ respectively. 10 µM acetaminophen was added to the test mixture, under the prediction that this species may be present in tears at a relative dilution ratio similar to blood glucose, if the patient treatment included this drug.
2.2.3 **Human Tear Sample Acquisition**

Testing of nine fasting, non-diabetic adult volunteers was conducted in the clinical research laboratory of EyeLab Group under the direction of Dr. Bruce Cohan, M.D. (ophthalmologist). A technician viewed the eye, which was not anesthetized, through a slit lamp microscope at ×16 magnification. Separate tear samples were obtained from the lacrimal lake via hand-held 1 µL disposable glass capillary tubes (micro-pipettes “Microcaps” Drummond Scientific Co., Broomall, PA.) from the right and then the left eye of each subject without intermission. Each subsequent set of samples was obtained after ~20 min intervals. Tear sample pairs (from each eye) were obtained during an ~3-4 h session. For some subjects, samples were obtained on multiple days.

2.2.4 **Human Tear Sample Glucose Measurement**

A micro tear bubble, produced at the end of the capillary tube by application of gentle force on a rubber bulb dispenser attached to the other end of the capillary tubes, was wicked from the tube into a test-strip (ACCU-CHEK Aviva Plus, LOT #s: 491361, 490702, Roche Diagnostics, Indianapolis, IN) attached to a potentiostat for immediate analysis of glucose concentration. For human tear measurements, average current values over a 10 s period were recorded starting at the 5 s time-point after calibrant or sample introduction were used to prepare the calibration curve and quantitate tear values, respectively.

2.3 **Results and Discussion**

2.3.1 **Sensitivity Analysis of Glucometer Strips**

Table 2.1 lists the commercially manufactured glucometer strips evaluated in this research along with the enzyme and mediator employed in each device, the LOQ (µM), and the $R^2$ values obtained
from preliminary studies. The working and reference/counter electrodes for each strip were identified from the manufacturers’ patent descriptions and/or the manuals supplied with the strip and/or meters. Strips from each manufacturer were tested with the standard glucose solution for 6 min at an applied voltage of +400 mV vs. Ag/AgCl (n = 5) at physiological temperature (37 °C). For these preliminary studies, a calibration graph (0 - 100 µM) with the greatest degree of linearity was used to obtain the LOQ by recording the ten second average of current starting at the 60 s time-point after solution was wicked into the strips.

Figures 2.1 show representative current vs. concentration data for several of the commercial strips tested. Standard deviations of the blank and the two glucose standards (50 and 100 µM), are large with high LOQ values (10x standard deviation for the blank) for the strips that employ FAD/NAD-dependent GDH or GOD (glucose oxidase) as the glucose-selective enzyme: Abbott, Bayer, and

Table 2.1. Enzyme and mediator combinations in commercial glucometer strips and preliminary assessment of detection capability at low level glucose concentrations

<table>
<thead>
<tr>
<th>Company</th>
<th>Product</th>
<th>Enzyme</th>
<th>Mediator</th>
<th>LOQ (µM)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott</td>
<td>FreeStyle Lite®</td>
<td>FAD-GDH</td>
<td>Osmium</td>
<td>470</td>
<td>0.9999</td>
</tr>
<tr>
<td>Abbott</td>
<td>Precision Xtra®</td>
<td>NAD-GDH</td>
<td>Phenanthroline Quinone</td>
<td>2040</td>
<td>0.6493</td>
</tr>
<tr>
<td>Bayer</td>
<td>CONTOUR®</td>
<td>FAD-GDH</td>
<td>Potassium Ferricyanide</td>
<td>287</td>
<td>0.9711</td>
</tr>
<tr>
<td>Johnson &amp; Johnson</td>
<td>OneTouch® Verio™</td>
<td>FAD-GDH</td>
<td>Potassium Ferricyanide</td>
<td>374</td>
<td>0.9931</td>
</tr>
<tr>
<td>Johnson &amp; Johnson</td>
<td>OneTouch® Ultra® Blue</td>
<td>GOD</td>
<td>Potassium Ferricyanide</td>
<td>954</td>
<td>0.3879</td>
</tr>
<tr>
<td>Roche Diagnostics</td>
<td>ACCU-CHEK® Aviva Plus</td>
<td>PQQ-GDH</td>
<td>Nitosoaniline Derivative</td>
<td>45</td>
<td>0.9923</td>
</tr>
<tr>
<td>Nipro Diagnostics</td>
<td>TRUEtest®</td>
<td>PQQ-GDH</td>
<td>N/A</td>
<td>100</td>
<td>0.9541</td>
</tr>
</tbody>
</table>

LOQ and R² from calibrations (0 - 100 µM) of glucose solution at average current recorded for 10 s starting 60 s following sample introduction (37 °C, +400 mV applied between working and counter electrodes; n = 5).
Johnson & Johnson strips (see Table 2.1). Similar results were obtained with other strips which do not utilize PQQ-dependent GDH (data not shown). Thus, all of these strips show inadequate sensitivity at glucose concentrations that mimic tears.

In contrast, strips manufactured with PQQ-GDH, by Roche Diagnostics and Nipro Diagnostics, exhibit far greater precision and linearity in the 0 - 100 µM glucose range. The lower LOQ values reported in Table 2.1 for these strips indicates better reproducibility of measured currents for the blank solution and standard glucose solutions for the average current recorded over a 10 s period beginning at the 60 s time-point after sample introduction, with the LOQs for these two strips approaching the lower range required for tear measurements.

Initial screening of the glucometer strips indicates that strips utilizing the PQQ-GDH enzyme with gold working and reference/counter electrodes exhibit the best performance at lower-end glucose

**Figure 2.1.** Calibration data for 0, 50 and 100 µM standard glucose solution using 10 s average current recorded starting at the 60 s time point after sample introduction (at 37 °C, applied voltage to working electrode = +400 mV) for several different commercial glucometer strips: (A) FreeStyle Lite® by Abbott; (B) Precision Xtra® by Abbott; (C) CONTOUR® by Bayer; and (D) OneTouch® Ultra® Blue by Johnson & Johnson. Error bars are standard deviations (S.D.) for n = 5 measurements.
concentrations, as compared to other strips. Consequently, the Nipro and Roche strips were further tested in more extensive detail for the effects of temperature and interfering species, as well as the influence of applied voltage to the working electrode and the timing of the current measurement after sample application. Figure 2.2A illustrates the current response of Roche strips over the initial 15 s after 0, 50, and 100 µM glucose standards are wicked into the strips, with an applied potential of +400 mV. The 10 s average current values recorded at 5 s after sample introduction yields calibration data (Figure 2.2B), equivalent to or better (higher current) than data obtained at the average current measured 60 s after sample application. Under these conditions, the LOQ for the Roche strip is 44 µM (n = 5). Similar results were obtained with Nipro strips (data not shown), with a LOQ of 40 µM (n = 5).
For tests at room temperature (Figure 2.2C and 2.2D), when the potential applied to the working electrode of the strips was changed to +300 mV and current averaged beginning at the 5 s time-point after sample application, the LOQs for the Roche and Nipro strips were improved to 3 and 17 µM, respectively (n = 5). Better glucose detection limits were observed for both ACCU-CHEK® Aviva Plus (Roche Diagnostics) and TRUEtest® (Nipro Diagnostics) strips, which may result from the lower $K_M$ value for glucose with PQQ-dependent glucose dehydrogenase$^{33-37}$ as compared to the other enzymes that catalyze glucose oxidation. The lower $K_M$ results in an increase of the rate of reaction and, therefore, increasing current from oxidation of reduced mediator at the working electrode, at lower glucose concentrations.

### 2.3.2 Selectivity Analysis for Glucometer Strips

<table>
<thead>
<tr>
<th>ACCU-CHEK® Aviva Plus</th>
<th>Calculated [Glucose] in Interference Solution based on Calibration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard [Glucose] in Interference Solution (µM)</td>
<td>+100 mV</td>
</tr>
<tr>
<td>25</td>
<td>28 ± 0</td>
</tr>
<tr>
<td>50</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>75</td>
<td>75 ± 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TRUEtest®</th>
<th>Calculated [Glucose] in Interference Solution based on Calibration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard [Glucose] in Interference Solution (µM)</td>
<td>50 mV</td>
</tr>
<tr>
<td>25</td>
<td>211 ± 21</td>
</tr>
<tr>
<td>50</td>
<td>150 ± 16</td>
</tr>
<tr>
<td>100</td>
<td>229 ± 14</td>
</tr>
</tbody>
</table>

Comparison of selectivity test results for ACCU-CHEK® Aviva Plus and TRUEtest® strips calibrated with standard glucose solutions and tested for measuring standard glucose solution with interfering species present (10 µM acetaminophen, 100 µM ascorbic acid, 100 µM uric acid) at the given applied voltages.
The selectivity of ACCU-CHEK® Aviva Plus and TRUEtest® strips over interferences was tested with glucose solutions in the presence of interfering species that may be found in tears (10 µM acetaminophen, 100 µM ascorbic acid, and 100 µM uric acid) (see Table 2.2). The ACCU-CHEK® Aviva Plus strips with three concentrations of glucose (25, 50, 75 µM), were tested with interferences at applied voltages in the range of +100 - 300 mV (n = 3) after obtaining calibration currents for glucose only in PBS buffer over this same range. The experiment was also conducted with the TRUEtest® strips with glucose concentrations of 25, 50, 100 µM and the same interferences but at applied voltages in the range of +50 - 300 mV (n = 5) to find the optimal potential for best selectivity.

Figure 2.3 shows the calibration data for the ACCU-CHEK® Aviva Plus and TRUEtest® strips in the range of 0 - 100 µM, at RT with different applied voltages testing glucose solutions containing....
interferences. Table 2.2 shows data with Roche and Nipro strips calibrated in standard glucose solution and then tested with known glucose solutions containing interfering species at the applied voltages. As shown in Figure 2.3 and Table 2.2, while Nipro strips exhibit good low-end reproducibility and detection limits toward glucose with applied voltages of +50, 100, 200, and 300 mV, large interference occurs at all applied potentials, yielding very large errors in glucose values for samples containing the three interfering species. In contrast, for Roche strips, at all applied potentials, the presence of interfering species yielded relatively small errors in measurement of glucose at levels < 100 μM.

Figure 2.4 shows the amperometric response to ACCU-CHEK® Aviva Plus strips with PBS solution (pH 7.4, 0 μM glucose, no interference) and glucose (25, 50, 75 μM) in interferences (10 μM acetaminophen, 100 μM ascorbic acid, 100 μM uric acid) for 15 s (at +150 mV, n = 3). The
change in current between 25, 50, and 75 µM glucose in the presence of interfering species is reproducible and displays good linearity (inset).

Figure 2.3 shows the effect of applied voltage for the ACCU-CHEK® Aviva Plus strips on the calibration data over the 1-100 µM glucose concentration range. Table 2.2 reports errors of ≤ 15% at all applied voltages; however, voltages of 150 mV and 200 mV tend to yield lower percent errors. The LOQ for both +150 mV and +200 mV applied voltages are 9 µM and 18 µM, respectively (n = 3). Since triplicate measurements are insufficient to yield true standard deviations and, in turn, obtain “true” LOQs, additional measurements were made at +150 mV and +200 mV, which resulted in LOQ values of 16 µM and 20 µM, respectively (n = 9). The optimal applied potential for the Roche Strips was found to be +150 mV.

It appears that the Roche ACCU-CHECK strips exhibit dramatically enhanced selectivity versus the Nipro strips which utilize the same PQQ-dependent GDH enzyme largely in part to the specific mediator utilized on the strip. Different mediators exhibit variable reactivity to species such as ascorbic and uric acid, which are easily oxidized. Indeed, the ACCU-CHECK strips utilize a nitrosoanaline derivative, which may be highly resistant to reduction by the ascorbic acid and uric acid species. It is, however, unknown which mediator is employed within the Nipro TRUE-Test strips.

2.3.3 Selectivity Analysis for Different Lots of Glucometer Strips
Preliminary testing revealed lot-to-lot variation in the Roche strip’s ability to differentiate glucose from interfering species. For each lot of strips, Roche provides an electronic calibration code
device for insertion into the associated glucometer (for blood measurements), suggesting some differences in the amperometric behavior from lot-to-lot. Therefore, the selectivity of eleven lots of these strips was tested at the optimal applied voltage of +150 mV (n = 3). The calculated errors in the presence of interferences in six of these lots are shown in Table 2.3. Nipro employs a “no-coding” technology in that insertion of the strip itself calibrates the meter. Nipro strips were not tested due to poor selectivity over interferences (see Table 2.2).

Table 2.3 shows errors for all six lots of Roche strips of ≤ 20 % with an applied voltage of +150 mV for all measurements, except the values for lot #491390 at 75 µM glucose. It is not clear why this lot behaved differently at this one higher concentration of glucose. However, differences in interference errors may exist from lot to lot, and thus only certain lots of the Roche strips may yield reliable glucose measurements in tears. Some manufacturing differences can surely exist from lot-to-lot that would give rise to such performance variation. Slight variations in the thickness of the reagent layer, working electrode area, and amount of mediator may not greatly

Table 2.3. Selectivity test results for six lots of ACCU-CHEK® Aviva Plus strips at +150 mV vs. internal strip counter electrode

<table>
<thead>
<tr>
<th>ACCU-CHEK® Aviva Plus</th>
<th>Calculated [Glucose] with Interferences (µM)</th>
<th>Calibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard [Glucose] in Interference Solution (µM)</td>
<td>Lot# 490702</td>
<td>Lot# 491678</td>
</tr>
<tr>
<td>25</td>
<td>27 ± 1</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>50</td>
<td>58 ± 2</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>75</td>
<td>81 ± 3</td>
<td>68 ± 1</td>
</tr>
</tbody>
</table>

*Comparison of selectivity test results for six different lots of ACCU-CHEK® Aviva Plus strips. Each lot was calibrated with standard pure glucose solutions and tested with the glucose standards containing interferences (10 µM acetaminophen, 100 µM ascorbic acid, 100 µM uric acid) at +150 mV applied voltage (n = 3).*
influence glucose measurements in the range of normal blood glucose concentrations, but could cause more discernible effects in the normal, much lower range of tear glucose concentrations.

2.3.4 Preliminary Assessment of Roche Strips for Measurements of Glucose in Human Tears

Two lots of ACCU-CHEK Aviva Plus strips were determined to possess suitable sensitivity and selectivity to measure glucose in human tear samples. Calibrations of both lots were made in the range of 5 - 100 µM glucose. LOQs were 7 and 4 µM, respectively (n = 3). Selectivity tests for these lots exhibited errors of ≤ 14 % (n = 3) for 25, 50, and 75 µM glucose in the presence of 10 µM acetaminophen, 100 µM ascorbic acid, and 100 µM uric acid. Tear samples obtained from n = 9 fasting, non-diabetic human subjects were collected in 1 µL micro capillary tubes. None of the measured glucose concentrations in human tear samples were found to be below the limit of detection (LOD) of 2 and 1 µM, respectively (n = 3 for each lot of strips). Table 2.4 lists the average, S.D., median, and range of all measured fasting tear glucose concentrations from each

Table 2.4. Summary of fasting human tear glucose measurements with ACCU-CHEK Aviva Plus strips for n = 9 non-diabetic volunteers.

<table>
<thead>
<tr>
<th>Fasting Tear Glucose Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
</tr>
<tr>
<td>Eye</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>S.D.</td>
</tr>
<tr>
<td>n</td>
</tr>
</tbody>
</table>

Comparison of selectivity test results for six different lots of ACCU-CHEK® Aviva Plus strips. Each lot was calibrated with standard pure glucose solutions and tested with the glucose standards containing interferences (10 µM acetaminophen, 100 µM ascorbic acid, 100 µM uric acid) at 150 mV applied voltage (n = 3).

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subject’s right and left eyes. Assuming a normal Gaussian distribution for each subject, a total of 7 outliers were identified and excluded from the 163 tear glucose measurements via the Grubbs’ outlier test. These outlier values could have resulted from small perturbations induced by the subject rubbing his/her eye in advance and anticipation of tear acquisition or by excess pressure applied to the surface of the eye during the tear acquisition process using the micropipette. In both instances, tiny volumes of the higher glucose levels within tissue or blood capillaries within the eye could enter the tear fluid, temporarily increasing tear glucose values.

Table 2.5 provides the range and median of fasting human tear glucose levels found in this study, and those reported by Asher and coworkers using LC-ESI-MS.\textsuperscript{23} With comparable numbers of measurements by the EyeLab and Asher groups, the range of values and the median values found for human tear glucose in non-diabetic subjects for the two data sets are quite comparable, suggesting that the simple glucometer-based method provides tear glucose values that are quite similar to the far more complex LC-MS method.

2.4 Conclusions
Herein, we have demonstrated that test-selected lots of commercially available ACCU-CHEK® Aviva Plus glucometer strips manufactured by Roche exhibit the sensitivity and selectivity

<table>
<thead>
<tr>
<th></th>
<th>Range (µM)</th>
<th>Mean (µM)</th>
<th>Median (µM)</th>
<th>n (Subject)</th>
<th>n (Measurement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EyeLab Group</td>
<td>5 - 148</td>
<td>47</td>
<td>43</td>
<td>9</td>
<td>156</td>
</tr>
<tr>
<td>Asher</td>
<td>7 - 161</td>
<td>N/A</td>
<td>28</td>
<td>25</td>
<td>148</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data excludes outliers identified by Grubbs’ test.
necessary to measure low levels of glucose in human tears using sample volumes of <1 µL. Preliminary measurements of tear glucose using these strips in non-diabetic volunteers correlate well with those reported by Asher with research laboratory LC-MS instrumentation. The future clinical potential for diabetes management of tear glucose testing with glucometer strips will require thorough evaluation in human subjects including responses to the full range of blood glucose levels during hyperglycemia and, especially, hypoglycemia. The practicality of near-patient and self-patient tear sample acquisition must also be addressed for safety and efficacy (i.e., incidence of and recovery from perturbation of the sampling site), prior to clinical employment of this measurement method. Certainly, the use of readily-available glucometer test strips in conjunction with a high current sensitivity patient-side measurement system (meter) will greatly aid in carrying out such required research studies.

2.5 References

(2) Hones, J.; Muller, P.; Surridge, N. *Diabetes Technol. Ther.* **2008**, *10*, S10.
CHAPTER 3

Origin of Low Detection Limit and High Selectivity of Roche Accu-Chek Test Strips that Enables Measurement of Tear Glucose Levels

Cha, K.H.; Qin, Yu.; Meyerhoff, M.E. Electroanalysis 2015; 27: 670-676 *

3.1 Introduction

The World Health Organization reports that 347 million people worldwide have diabetes\(^1\) and projects that diabetes will be the 7th leading cause of death in 2030,\(^2\) despite the advancing modern medical science and technology.\(^3,4\) Tight glycemic control is essential to prevent or reduce life-threatening hyper- and hypoglycemic crises causing serious long-term complications.\(^5\) Using a personal blood glucometer with an enzymatically-active disposable test strip, enables diabetic patients to monitor their glucose levels by obtaining a small drop of capillary blood from their fingertips or forearms with a conventional lancet. While minimum of four measurements of glucose per day is recommended to maintain normal levels of glucose in blood,\(^6\) up to eight checks per day are recommended for

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* The key experiments of this chapter were conducted by Kyoung Ha Cha, with help from Dr. Yu Qin in providing the nitrosoaniline derivative.
children and adolescents with Type 1 diabetes on insulin treatment.\textsuperscript{7} Pricking the finger tip for many times a day can limit patient compliance and develop massive carring/callous formation and loss of sensibility/perception hindrance. Consequently, it may lead to suboptimal blood glucose control and induce hypoglycemia. Therefore, the development and availability of a pain-free glucose measuring device could greatly increase patient compliance and maintain optimal glucose control.

Tear glucose measurements have been suggested as an alternative method to monitor glucose levels. Tear glucose measurement techniques require a low micromolar LOQ, high selectivity over potential interferences such as ascorbic acid, uric acid and acetaminophen, and measurement within microliter or sub-microliter sample volume. Since several studies have shown that there may be a clinically useful correlation between blood and tear glucose levels,\textsuperscript{7-9} more detailed studies are necessary to confirm the potential utility of tear glucose measurements to help maintain optimal glucose control. Chapter 2 of this dissertation and Cha et al. recently reported\textsuperscript{10} that Roche’s Accu-Chek blood glucose test strips are capable of glucose level measurements in tear samples with adequate sensitivity (0.127 nA/µM) and selectivity with ≤13 % error (n = 3) for 25, 50, and 75 µM glucose in the presence of 10 µM acetaminophen, 100 µM ascorbic acid, and 100 µM uric acid. In this previous work, the Roche test strips were used for measurement of glucose concentration in tears from n = 9 normal (non-diabetic) fasting human subjects and reported glucose values were within the range of 5-148 µM (mean = 47 µM, median = 43 µM), a similar range to that reported by Asher and co-workers using an LC-MS technique.\textsuperscript{8} Herein, we utilize bulk phase solution experiments with different enzymes, strip-based screen printed electrode materials and mediators to explain why the Roche test strips exhibit such excellent detection limits.
and selectivity compared to all other strips examined in our earlier studies.\textsuperscript{10} Results indicate that the unique combination of pyrroloquinoline quinone dependent glucose dehydrogenase (PQQ-GDH) as the active enzyme and a nitrosoaniline derivative as the electron transfer mediator are the key components of this test strip that yield the required sensitivity and selectivity to accurately measure low glucose levels in tear fluid.

3.2 Experimental Section

3.2.1 Chemicals and Reagents

D- (+) -Glucose, glucose oxidase from \textit{A. niger} (149.8 U/mg), sodium chloride (NaCl), potassium ferricyanide(III) (K\textsubscript{3}Fe(CN)\textsubscript{6}), potassium chloride (KCl), sodium phosphate dibasic (Na\textsubscript{2}HPO\textsubscript{4}), potassium phosphate monobasic (KH\textsubscript{2}PO\textsubscript{4}), ascorbic acid, uric acid sodium salt, and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO). FAD (flavin adenine dinucleotide)-dependent glucose dehydrogenase from Aspergillus sp. (1180 U/mg) was obtained from Sekisui Enzymes (Lexington, MA). PQQ-dependent glucose dehydrogenase (808 U/mg) was a product of Toyobo Enzyme (Osaka, Japan). O-methoxy-[N,N-bis-(2-hydroxyethyl)]-p-nitrosoaniline (nitrosoaniline derivative) (see Fig. 3.1) was generously donated by PharmaBlock R&D Co. Ltd (Nanjing, China).

A four-channel Biostat (ESA Biosciences Inc., Chelmsford, MA) was used to measure the amperometric response to glucose solutions (0-5 mM) containing enzymes, mediators and/or potential interfering species (easily oxidizable species at applied potentials) using an applied potential of +150 mV. For testing electrodes with different materials (palladium, gold and carbon), commercially available glucose test strips from Bayer, Roche Diagnostics and Abbott were
purchased over the counter and the top housing layers were removed to expose the electrochemical sensing sites. Each of these strips use palladium, gold and carbon as the working electrode material, respectively. The exposed surfaces of the strips were then washed thoroughly with deionized water and ethanol to remove any deposited chemical reagents on the surface of electrodes before use in bulk phase solution experiments.

A 1.5 M stock solution of glucose, and 25 mM stock solutions of ascorbic acid, uric acid and acetaminophen were made in phosphate saline buffer (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 2.0 mM KH$_2$PO$_4$, adjusted pH to 7.4 with 1.0 M NaOH. Standard solutions were prepared by dilution of the stock solutions using PBS solution for calibrations of glucose. Solutions of 0.5 mM ascorbic and uric acid and 0.2 mM acetaminophen (freshly made each day) were used to examine the cumulative effect of these potential interference species on the accuracy of glucose measurements. The reported average levels of ascorbic and uric acid in tears are 20 ± 6.2 and 68 ± 46 µM, respectively.$^{11-12}$ A 0.2 mM acetaminophen solution was also tested assuming that this species may be present in tears at a relative dilution ratio similar to blood

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**Figure 3.1.** Structures of (a) ferricyanide and (b) O-methoxy-[N,N-bis-(2-hydroxyethyl)]-p-nitrosoaniline that were examined as electron transfer mediators in this work.
glucose, if the patient was using this drug. Stock solutions of the various enzymes and mediators were made in PBS solution in the same way.

3.2.2 Electrochemical Measurements

Various glucometer strips for which the reagents layers were removed and the electrodes first exposed and cleaned were used as biamperometric detectors in bulk solution phase experiments. Each of the strips has two identical electrodes that are utilized for glucose detection with one electrode employed as the working electrode (apply positive voltage to oxidize reduced form of mediator formed from enzymatic oxidation of glucose and/or reduction of mediator by potential interferent species that can directly reduce the mediator) and the other serving as the pseudo reference/counter electrode. The cleaned and uncovered electrode ends of the various strips were placed in 5 mL of PBS buffer within a vial and a small magnetic stir bar was utilized to mix the solution. Different mediators (e.g., ferricyanide or the nitrosoaniline derivative (see Fig. 3.1)) alone or in combination with different glucose oxidizing enzymes (at near equivalent activity in term of Units/mL) were added to the PBS solution for testing response to ascorbic acid, uric acid and acetaminophen, as well as to different concentrations of glucose. The Biostat electrochemical analyzer was used to apply the indicated voltage between the two electrodes of the strips and to monitor the resulting current as a function of time after addition of glucose or test interference species.

3.3 Results and Discussions

3.3.1 Sensor Configuration for Tear Glucose Measurement
A schematic of a generic blood glucometer test strip when used for monitoring glucose in tear fluid is illustrated in Figure 3.2. An assortment of different glucose-oxidizing enzymes have been employed to prepare conventional glucose biosensors for measuring glucose concentration in whole blood. Glucose oxidase (GOX) has been widely used to catalyze oxidation of glucose on or close to the electrode surface, which can be followed by amperometric monitoring of liberated \( \text{H}_2\text{O}_2 \) or the consumed \( \text{O}_2 \). However, monitoring peroxide production when using GOX requires a high applied potential (\(+600\) mV),\(^{13}\) at which potential interferents can also be easily oxidized. Fortunately, via the aid of electron transfer mediators the applied potential required can be significantly lowered to partially reduce the interference problem. Indeed, efficient electron transfer mediators such as ferrocene and its derivatives were originally used for the enzymatic reaction catalyzed by GOX and applied in Abbott’s Medisense Precision Q.I.D. blood glucometer test strips back in the early 1990s. However, since then, other mediators including potassium ferricyanide, osmium complexes and nitrosoaniline derivatives have become the mediators of choice and are the ones most widely used in current commercial blood glucometer test strips. In

![Figure 3.2. Schematic of blood glucometer test strip and its potential application for monitoring glucose levels in a tiny volume of tear fluid. In addition to gold and carbon electrodes utilized in a biamperometric sensing mode, palladium and other metals have been utilized.](image)

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fact, several commercial strips (e.g., Johnson and Johnson One Touch, Bayer Contour, etc.) utilize potassium ferricyanide as the mediator in the strip configuration illustrated in Figure 3.2. Beyond glucose oxidase, two different types of glucose dehydrogenase (GDH) enzymes are also often employed to create blood glucometer strips; FAD-dependent GDH, and PQQ-dependent GDH. The Roche test strips that were found to be so useful for tear glucose measurements utilize a unique combination of gold working/counter electrodes, PQQ-GDH as an active enzyme and a nitrosoaniline derivative (see Fig. 3.1) as an electron transfer mediator. This strip is able to selectively measure low concentrations glucose found in tears in the presence of electroactive interferents. An optimal applied voltage of +150 mV between the two gold electrodes within the Roche strips for calibration over the 1-100 µM glucose concentration range was found to yield the lowest % errors from such potential interferent species.¹⁰

3.3.2 Selectivity Analysis

To better understand the enhanced selectivity over potential interferences of the Roche Accu-Chek glucometer strips vs. other common brands when applied for tear measurements, we first examined the response of interfering species (ascorbic acid, uric acid and acetaminophen) on three different electrode strip materials (palladium, gold and carbon) in terms of the steady-state amperometric responses in the presence of two different electron transfer mediators, potassium ferricyanide and the nitrosoaniline derivative (Fig. 3.1), in bulk solution experiments. As stated above, potassium ferricyanide is the most widely used electron transfer mediator at present, and thus it was chosen to be compared directly to the nitrosoaniline derivative. Our aim was to determine the ideal combination yielding the least current from oxidation of the potential interferents. An applied potential of +150 mV between the two strip electrodes was chosen because it is the optimal
potential found previously to achieve the highest selectivity for glucose for the Roche test strips with nitrosoaniline derivative as an electron transfer mediator. Further, this applied potential is close to the +200 mV value that is generally used by the commercial glucometer devices in their intended operation protocols for test strips employing potassium ferricyanide as an electron transfer mediator.

As shown in Figure 3.3, a large increase in current is observed on gold-printed electrodes when 0.5 mM ascorbic acid is added at the 80 s mark to the stirred PBS bulk solution (pH 7.4) for sensor strips with either potassium ferricyanide or nitrosoaniline derivative as the redox mediators (+150
It should also be noted that the level of currents observed from the addition of ascorbic acid is much greater when ferricyanide is selected as the mediator vs. when an equivalent concentration of nitrosoaniline derivative is employed in the bulk solution (10 mM) under the exact same experimental conditions. However, when the nitrosoaniline derivative is employed (Fig. 3.3b), 0.5 mM ascorbic acid is the only potential interferent species yielding a significant current at +150 mV applied potential (primarily due to reduction of the mediator by ascorbate). As shown in Figure 3.3b, 0.5 mM uric acid and 0.2 mM acetaminophen are not electro-oxidized at all with 10 mM nitrosoaniline derivative in PBS solution. In contrast, when potassium ferricyanide is selected as the mediator, relatively large responses from uric acid and acetaminophen are observed at an applied potential

![Figure 3.4. Comparison of dynamic amperometric responses (at room temperature; applied potential = +150 mV) from the interferents (0.5 mM ascorbic acid (blue), 0.5 mM uric acid (red) and 0.2 mM acetaminophen (green)) in the presence of 10 mM potassium ferricyanide (a, c) or 10 mM nitrosoaniline derivative (b, d) in PBS (pH 7.4, 0 μM glucose) on palladium (a, b) and carbon electrode (c, d). Background response from 10 mM potassium ferricyanide (a) and nitrosoaniline derivative in PBS solution is recorded between 0-20 s for a, b, and d and 0-80 for c. Response from interferents are recorded afterwards.](image)
of +150 mV vs. the counter electrode (Fig. 3.3a). This behavior is also observed for palladium and carbon-printed electrodes (Fig. 3.4). When using potassium ferricyanide as mediator, all three types of electrodes (palladium, gold and carbon) exhibit a significant current increase in the presence of potential interferents, while 0.5 mM uric acid and 0.2 mM acetaminophen do not yield an increase in anodic current with a nitrosoaniline derivative mediator. It is well documented that ascorbic acid can react with the mediators in their oxidized form to form reduced mediator which can then be oxidized electrochemically. Our results indicate that the nitrosoaniline derivative is much more inert to these interfering species than potassium ferricyanide, making the nitrosoaniline derivative a better choice for electron transfer mediator to yield more selective glucose measurements in blood as well as in tear fluid. Initial screening of all possible combinations of different electrode materials and mediators indicates that the use of gold working/counter electrodes along with the nitrosoaniline derivative is the only combination that exhibits the optimal selectivity over potential interferents in the bulk phase experiments with the commercially-branded, printed electrodes test strips. Consequently, this combination was utilized in further studies to measure low-end glucose concentrations using different glucose oxidizing enzymes as catalysts for detection of glucose.

3.3.3 Sensitivity Analysis

To this end, three different enzymes used for glucose measurements were examined in the bulk phase experiments using biamperometric detection with various glucometer strip electrodes. The enzymes tested included: glucose oxidase and glucose dehydrogenases in combination with two different cofactors/prosthetic groups, flavin adenine dinucleotide (FAD) and pyrroloquinoline quinone (PQQ). The enzymes were added to the PBS buffer alone with either potassium
ferricyanide or nitrosoaniline derivative, and the biamperometric response toward additions of glucose concentrations in the range of 0-5 mM were recorded. The response curves toward glucose were obtained initially in PBS without any interfering species present. As shown in Figure 3.5a, with 125 U/mL FAD- or PQQ-dependent GDHs, coupled with 20 mM potassium ferricyanide in the test solution and gold electrodes as the biamperometric detectors (at an applied potential of +150 mV), significant amperometric signals are observed for low-end glucose concentrations in the range of 0-0.2 mM. In contrast, when using GOX at the same U/mL concentrations, there is very poor sensitivity to changes in glucose concentrations, with amperometric response observed only in the higher concentrations range (≥0.5 mM). In the case of the nitrosoaniline derivative (Fig. 3.5b), only the PQQ-GDH catalyst yields significant amperometric response to glucose in the low concentration range required for tear glucose measurements. It is important to note that 20 mM of both mediators was used for these experiments, instead of 10 mM, in order to have a sufficient level of mediator necessary to oxidize high-end glucose concentrations.

The far superior glucose sensitivity observed in the above experiments when using both FAD- and PQQ-dependent GDH may relate to their lower Michaelis-Menten constant $K_M$ values, compared to the other enzymes that catalyze glucose oxidation. The lower $K_M$ results in an increased rate of reaction at a lower glucose level and, therefore, increased catalytic current from oxidation of the reduced mediator at the working electrode for lower glucose concentrations. Indeed the Michaelis-Menten constant $K_M$ values toward glucose of GOX, FAD-GDH, NAD-GDH, and PQQ-GDH are reported to be 27, 0.46, 10 and 0.38 mM, respectively. Hence, there is a significantly lower $K_M$ value for the two enzymes that exhibit the most sensitive response to glucose levels in the bulk solution phase experiments. Further, from the patent literature, it is known that Roche utilizes a
recombinant form of PQQ-GDH in order to avoid interference from maltose that can cause errors with blood glucometer devices that utilize PQQ-GDH as the active enzyme. Hence, it is possible that the recombinant form of PQQ-GDH utilized in the Accu-Chek strips may have an even lower $K_M$ compared to the 0.38 mM value reported for the native enzyme.

Figure 3.5. Dynamic amperometric response (at room temperature; applied potential = +150 mV) for changes in glucose concentration (0-5 mM) in the presence of (a) 20 mM potassium ferricyanide and (b) 20 mM nitrosoaniline derivative in PBS solution (pH 7.4) with 125 U/ml glucose oxidase (GOX), FAD- (FAD-GDH), and PQQ-dependent glucose dehydrogenase (PQQ-GDH). The activities of the enzymes were 149.8 U/mg, 1180 U/mg and 808 U/mg, respectively. Insets: Current response from low-end glucose concentrations (0-0.2 mM). Within the figure, the indicated concentrations represent the total concentration of glucose at each arrow point in time.
Although use of FAD- and PQQ-dependent GDHs in combination with potassium ferricyanide as the electron transfer mediator exhibits very good sensitivity for low concentration detection of glucose, the use of potassium ferricyanide is undesirable due to the considerable amount of interference current produced from ascorbic acid, uric acid, and/or acetaminophen oxidation of, and from this mediator (see Fig. 3.3a, above).

As stated above, when using nitrosoaniline derivative as the electron transfer mediator (Fig. 3.5b), PQQ-GDH produces the most sensitive response to glucose among all three enzymes tested, especially in the desired tear glucose measuring range (0-0.2 mM). With the nitrosoaniline derivative, GOX and FAD-GDH only yield amperometric response to glucose in the higher range of glucose levels (≥0.5 mM). It should be noted that for same enzyme, the sensitivity differs significantly between the different mediators. Our results indicate that for FAD-GDH, potassium ferricyanide is a better mediator than nitrosoaniline for facilitating the electron-transfer process. However, for PQQ-GDH, the use of the nitrosoaniline derivative is required to obtain enhanced sensitivity, and the selectivity over interfering species is far superior to the combination of FAD-GDH and potassium ferricyanide.

The rapid decrease in current after every glucose measurement for the combination of 20 mM nitrosoaniline derivative in PBS solution (pH 7.4) with 125 U/ml GOX and PQQ-GDH (as shown in Fig. 3.2b) that exhibit far more rapid response time, may be a result of adsorption of nitrosoaniline derivative onto electrode surface. When bare electrodes were used to measure current in 10 mM potassium ferricyanide/10 mM potassium ferrocyanide solution with an applied potential of +150 mV, steady currents are measured after ~5 minutes (solid lines in Fig. 3.6).
However, when the same electrodes are used to measure in 10 mM potassium ferricyanide/10 mM potassium ferrocyanide solution after measuring the 20 mM nitrosoaniline derivative, higher and unstable currents are obtained (dashed lines in Fig. 3.6) after 2 h with the same applied potential. This suggests that there may be an additional reaction between in 10 mM potassium ferricyanide/10 mM potassium ferrocyanide and the nitrosoaniline derivative that might have been absorbed onto electrode surface. Indeed, the reaction between 10 mM potassium ferricyanide/10 mM potassium ferrocyanide and the nitrosoaniline derivative with an applied potential of +150 mV yields currents in the range of 2-3 µA, but the 20 mM nitrosoaniline derivative itself yields only ~6 nA with the same applied potential (data not shown).

### 3.3.4 Optimization for Measurement of Low-end Glucose Level

The electrochemical response of the strips made with gold electrodes to glucose in the presence of ascorbic acid in the bulk solution was also examined using the optimal combination of PQQ-GDH...
and the nitrosoaniline derivative. The previous results (see Fig. 3.3) suggested that ascorbic acid still poses a problem and that the combination of PQQ-GDH, nitrosoaniline derivative, and gold electrode detection cannot avoid oxidation of ascorbic acid at an applied potential of +150 mV. As shown in Figure 3.7a, with 10 mM nitrosoaniline derivative present in solution, the glucose sensitivity at low concentrations is better in the presence of 0.1 mM ascorbic acid than 0.5 mM ascorbic acid. However, as also shown in Figure 3.7a, the interference effect from ascorbic acid can be partially minimized by controlling the concentration of the mediator, through which the reaction between a lower concentration of nitrosoaniline derivative (3.7 mM) and 0.1 mM ascorbic acid yields less anodic current than the reaction between 10 mM nitrosoaniline derivative and 0.1 mM ascorbic acid. Figure 3.7b shows the resulting glucose calibration curves for the gold-printed electrodes coupled with PQQ-GDH and 3.7 mM nitrosoaniline derivative (in bulk solution) in the absence and presence of 0.1 mM ascorbic acid. Although in the evaluation range of 0-50 μM glucose, the oxidation current from ascorbic acid is high, at the 50 μM glucose level, the current increase by the presence of 0.1 mM ascorbic acid is ca. 27 %, corresponding to +13 μM error in
the glucose measurement. At 200 µM glucose, the error from ascorbic acid is only 1%. However, in prior work with fully assembled Roche Accu-Chek strips, much lower errors were observed when ascorbate at 0.1 mM was combined with acetaminophen and uric acid as an interference solution in the presence of 50 µM glucose (combined error from interferences <13%). Since in the fully assembled commercial blood glucometer strip, the PQQ-GDH and nitrosoaniline derivative (1:2 by weight) are coated over the surface of the gold electrodes within a polymeric matrix containing thickeners stabilizers, detergents, and other adjunct materials, and the current is recorded after 5 s after wicking of the test samples into the strips, there may well be some partial mass transport rejection of ascorbic acid into the enzyme/mediator layer that further enhances selectivity compared to the homogeneous experiments reported in this work.

3.4 Conclusions

We have demonstrated that the ability of Roche Accu-Chek test strips to be used to measure µM levels of glucose without substantial interference from other redox active species results from the unique combination of using PQQ-GDH as the active enzyme in combination with nitrosoaniline derivative as an electron transfer mediator. The low $K_M$ value of this enzyme, coupled with the inability of uric acid and acetaminophen to reduce the nitrosoaniline mediator and yield electrochemical response in a biamperometric measurement mode, appear to be the key elements that provide the excellent LOQ value and selectivity of these strips. These findings suggest that any future efforts to fabricate specialized electrochemical glucometer type test strips, specifically for direct sampling of sub-microliter quantities of tear fluid from eyes, should utilize PQQ-GDH in combination with O-methoxy-[N,N-bis-(2-hydroxyethyl)]-p-nitrosoaniline as the reagents.
3.5 References


CHAPTER 4

Determination of Correlation Between Tear and Blood Glucose Levels of Human Subjects with Type 2 Diabetes Using Roche Accu-Chek Test Strip*

4.1 Introduction

As summarized in the preceding two chapters, the Roche Accu-Chek blood glucose test strip is unique in its ability to measure the very low levels of glucose that are present in normal human subjects.\textsuperscript{1-2} The mechanism that enables these strips to accurately measure such low levels of glucose without significant interference from common redox species has also been examined/determined.

Herein, we investigate the correlation between tear and blood glucose levels of Type 2 diabetic patients before and then over 1.5-h period after these patients consumed 50 g glucose in a tube of jelly. The measurements of the tear samples were made using the commercially available ACCU-CHEK Aviva Plus blood glucose test strips and a high sensitivity potentiostat, to definitively

* The key experiments of this chapter were conducted by Kyoung Ha Cha, with help from Dr. Taj Khan, M.D. in providing diabetic patients.
establish whether or not tear glucose is a potential noninvasive supplemental method to blood glucose measurements.

4.2 Experimental Section

4.2.1 Materials

D- (+)-glucose, sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na₂HPO₄), potassium phosphate monobasic (KH₂PO₄), L-ascorbic acid, uric acid, and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2 Sensitivity and Selectivity Analysis

To determine the sensitivity and selectivity of commercial glucometer strips, a four-channel Biostat (ESA Biosciences Inc., Chelmsford, MA) measured the amperometric response to glucose solutions (0-100 µM) and to glucose solution (50 µM) containing potential interfering species (easily oxidizable species at applied potentials). For testing of the commercial glucose strips, the reported applied potentials are versus the internal reference/counter electrode within the strips.

4.2.3 Human Tear Sample Acquisition and Analysis

Under an IRB approved protocol (through Rutgers University), fasting human subjects (n = 4) with type 2 diabetes were tested in New Jersey under the direction of Dr. Taj Khan, M.D. (ophthalmologist) who viewed the eyes of the human subjects, who were not anesthetized, through a slit lamp microscope at ×16 magnification. After separate tear samples were obtained from the lacrimal lake via hand-held 1-µL disposable glass capillary tubes (micropipets “Microcaps” Drummond Scientific Co., Broomall, PA.) from the left and then the right eye of each subject, subsequent blood glucose levels were measured without intermission using a blood glucometer. A
glucose challenge was introduced to the patients after the first set of samples were collected. This was accomplished by having the patients consuming 50 g glucose in a tube of jelly. From then, each subsequent set of samples were obtained with 30 min intervals for the next 90 min resulting in the total of 4 sets of tear collections per human subject. All human tear samples were measured using the same procedure described by Cha et al.\textsuperscript{1-2} with the ACCU-CHEK Aviva Plus test strips (Lot No. 496974, Roche Diagnostics, Indianapolis, IN). And all blood glucose levels were measured using the same strips with the paired commercial glucometer. For human tear measurements, 2 s average current values were recorded starting at the 2 s time-point after calibrant or sample introduction were used to prepare the calibration curve and quantitate tear values, respectively.

4.3 Results and Discussion

As shown in Figure 4.1a, a calibration of 0-100 µM glucose obtained by using the Roche test strips (Lot No. 496722) yields a LOD and LOQ of 7 and 22 µM (n = 3), respectively. The test strips also exhibit \( \leq 6.7\% \) error (n = 3) for measuring 50 µM glucose in the presence of potential interference species, including 10 µM acetaminophen, 100 µM ascorbic acid, and 100 µM uric acid. As shown in Figure 4.1b, a different lot of the test strips (Lot No. 496974) with an LOD and LOQ of 2 and 7 µM (n = 3) was evaluated to be used for human testing. Of note, calibration data for glucose used 2 s average current recorded starting at the 2-s time point after sample introduction with an applied potential of +150 mV. The method for averaging currents recorded is somewhat different from Chapter 2 but provides more accurate results. Error bars are SD for n = 3 replicate measurements. The reported average levels of ascorbic and uric acid in tears are 20 ± 6.2 and 68 ± 46 µM,\textsuperscript{32-33} respectively. A 5 mM stock solution of glucose was made in phosphate saline buffer
PBS solution containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, adjusted pH to 7.4 with 1 M NaOH. Standard solutions were prepared by dilution of this stock solution using PBS solution for calibrations of glucose at 0, 50, and 100 µM. Ten µM acetaminophen was added to the test mixture, assuming that this species may be present in tears at a relative dilution ratio similar to blood glucose, if the patient was using this drug.

Tear samples obtained from n = 4 fasting, type 2 diabetic human subjects were collected in 1 µL microcapillary tubes. As shown Figure 4.2, the chronological changes in tear and blood glucose levels of these 4 human subjects with type 2 diabetes clearly demonstrates a correlation. The

Figure 4.1. Calibration data for glucose using 2 s average current recorded starting at the 2 s time point after sample introduction with an applied potential of +150 mV. (a) 0-100 µM glucose calibration obtained by using test strips (Lot No. 496722). The limit of detection (LOD) and limit of quantitation (LOQ) are 7 and 22 µM, respectively. The test strips exhibit ≤6.7% error (n = 3) for 50 µM glucose in the presence of potential interference including 10 µM acetaminophen, 100 µM ascorbic acid, and 100 µM uric acid. (b) 0-400 µM glucose calibration obtained by test strips (Lot No. 496974), used for human testing. The LOD and LOQ are 2 and 7 µM, respectively. Error bars are SD for n = 3 replicate measurements.
lowest level readings were for the samples obtained prior to the patients consuming 50 g glucose in a tube of jelly. The excluded value (marked with *) could have resulted from small perturbations induced by the subject rubbing his/her eye in advance of tear acquisition or by excess pressure applied to the surface of the eye during the tear acquisition using the micropipet. In both instances, tiny volumes of the higher glucose levels within tissue or blood capillaries within the eye lid could enter the tear fluid, temporarily increasing tear glucose values. It is also important to note that the same trend in change tear glucose levels can be observed in the both eyes of each human subject and that the magnitude of the tear glucose levels differ significantly for each patient.

As shown in Figure 4.3, the tear and blood glucose levels yield moderate correlation ($R^2$) values of 0.6294, 0.6174, 0.5642, and 0.7021 for each human subject with Type 2 diabetes that was tested,
respectively. Due to this study being a preliminary human study for tear and blood correlation and the very first of its kind using this type of glucose sensing method, the correlation values obtained are a reasonably good fit based on the number of human subjects tested. However, future studies are needed to improve the correlation value. In addition, such significant differences in the magnitude of tear glucose levels for each of the 4 patients suggests that it is imperative to further investigate what factors dictate the ratio between blood and tear glucose levels from patient to patient. Whether this ratio is also influenced by the patient having or not having diabetes also must be examined.

4.4 Conclusion

In this chapter, the ACCU-CHEK Aviva glucometer test strips were used to measure glucose levels in tear fluids from 4 fasting human subjects with Type 2 diabetes before and over a 1.5 h period.
after these patients consumed 50 g glucose in a tube of jelly. Blood glucose values were also measured as similar time points as the tear measurements. A moderate correlation between tear and blood glucose levels was found for each patient, suggesting that measurement of tear glucose is a potential noninvasive supplemental method for diabetic patients to monitor blood glucose levels. However, the difference in magnitude of tear glucose levels between patients suggests that a longer-term analysis of each patient (more than 1.5 h) may be the key to definitively establish whether the correlation between tear and blood glucose levels is good enough to be clinically useful for such patients.

4.5 References

CHAPTER 5

Compatibility of Nitric Oxide Release with Implantable Enzymatic Glucose Sensors Based on Osmium (III/II) Mediated Electrochemistry

Cha, K.H.; Meyerhoff, M.E. ACS. Sens. 2017; 2(9): 1262-1266 *

5.1 Introduction

Tight glycemic control is essential to prevent or reduce life-threatening hyper- and hypoglycemic events that can cause serious long-term complications for critically ill patients.¹ Therefore, the development of continuous glucose monitoring systems to quantitate blood glucose levels intravascularly (IV) would greatly enhance glycemic control and, ultimately, improve patient outcomes, especially within intensive care units.²-⁶ FDA approved electrochemical continuous glucose sensors (e.g., commercial Dexcom, Medtronic, and Abbott glucose sensor systems) rely on measurements being made within the subcutaneous interstitial fluid, not within the blood stream. In most cases, frequent calibrations (once or twice per day) are required, by using a blood glucometer test strip and

* The key experiments of this chapter were conducted by Kyoung Ha Cha.
a finger-prick, to update the calibration between blood levels and the sensor’s output current. However, it is known that a significant lag time exists between interstitial and blood glucose levels (5-10 min). Further, devices implanted subcutaneously can suffer from a foreign body response (FBR), which can “wall off” the sensor, altering its sensitivity and potentially increasing the lag time to detect changes in blood glucose levels. Therefore, for patients in a hospital setting, implantation of continuous glucose sensing devices within the bloodstream would be convenient and advantageous.

To date, such continuous IV glucose sensors have not been successfully developed due, in part, to activation of platelets that can adhere to and form blood clots on the surface of the sensor (see Chapter 1). The presence of adhered, metabolically active platelets can change (lower) the local glucose levels at the surface of the device. To overcome this issue, many efforts have been made to reduce cell adhesion on the surface of implanted sensors and other IV devices by using various types of modified polymers, yet none have been successful.

Nitric oxide (NO) is endogenously produced from endothelial cells that line the inner walls of all healthy blood vessels and is known to prevent platelet activation and subsequent thrombosis, as well as serve as a potent antimicrobial/anti-biofilm agent. Therefore, one potential strategy to prevent thrombosis and infection associated with intravascular sensing devices is to utilize polymer coatings that release NO at physiological levels of ca. $0.5 \sim 4.0 \times 10^{-10} \text{ mol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$. Indeed, Yan et al. developed needle-type amperometric glucose sensors based on hydrogen peroxide detection with an NO releasing polymer and demonstrated that such sensors exhibit more accurate glucose measurements, as well as reduced thrombus formation over an 8 h period.
compared to control sensors when implanted in the veins of rabbits. Soto et al. also demonstrated the potential of NO release to enhance the analytical performance of subcutaneous glucose biosensors. Further, NO release coatings have also been successfully employed to improve the in vivo analytical accuracy of intravascular oxygen sensors. However, in the case of glucose sensors based conventional hydrogen peroxide detection (oxidation), devices with NO-releasing coatings that exhibit higher NO fluxes (desirable for more antithrombotic and antimicrobial activity) exhibit larger background currents in absence of glucose. This is due to the oxidation of NO at +0.65 V vs. Ag/AgCl at the surface of the underlying Pt/Ir working electrode of these sensors. Further, the level of this background current contribution decreases with time, as the NO flux decreases, thereby changing the low-end calibration curve for the sensor. Therefore, using a different glucose sensing method requiring a lower oxidation potential could further improve the compatibility of NO release coatings with implantable enzymatic glucose sensors.

In the 1990’s, Heller and coworkers introduced the concept of a wired-enzyme electrode that is now utilized by Abbott Laboratory in their subcutaneous, continuous glucose-monitoring system. Such an enzyme electrode is based on “wiring” glucose oxidase (GOX) to an electron-transfer osmium ion mediator, Os(bpy)$_2$:Cl$^{+/-}$ (osmium(II) complex with bipyridine), further complexed to poly(1-vinylimidazol) (PVI-Os). The potential required for oxidation of Os(II) to Os(III) is very low, <+400 mV vs. SCE, a potential that should avoid the additional background current from the oxidation of nitric oxide (to nitrate) at the inner working electrode, when NO release polymers are used to coat/contain the devices.
Herein, we demonstrate the compatibility of nitric oxide with implantable enzymatic glucose sensors based on the osmium (III/II) chemistry by fabricating NO-releasing osmium-mediated glucose sensors and testing them in both phosphate buffer (pH 7.4) and in vitro within heparinized whole porcine blood at 37 °C. Amperometric glucose response is preserved in the presence of continuous NO release from the surrounding NO-releasing polymer, suggesting that NO does not interact in any way with the Os(III/II) redox center of Pt/Ir working electrode, and thus does not alter the amperometric signals of such glucose sensing devices.

5.2 Experimental Section

5.2.1 Sensor Fabrication

Two fabrication methods were employed to prepare the two types of needle-type glucose sensors employed in this work. One design (Fig. 5.1a) is based on the configuration reported by Bindra et al.,24 Gifford et al.25 and Yan et al.19 One µL of 4.5 mg/mL PVI-Os (III) in methanol, 1 µL of 0.9 mg/mL glucose oxidase (Type VII, from aspergillus niger) in a mix of methanol and HEPES (1:1 wt%), and 1 µL of 0.3 mg/mL poly(ethylene glycol) diglycidyl ether (PEGDE) were dropped

[Diagram of sensor designs]

**Figure 5.1.** Designs of implantable (a) osmium-mediated glucose sensor and (b) NO-releasing osmium-mediated glucose sensor.
sequentially into a 1 mm cavity created on the side of an insulated platinum-iridium wire (see Fig. 5.1a). The sensor was subsequently rinsed with water and allowed to dry for 1 h. Then, the sensor was dip-coated with a thin layer of Nafion to enhance selectivity over potential anionic interferences such as ascorbic and uric acid, and then allowed to dry overnight in a 37 °C oven. The sensor was then loop-coated with ca. 1% (wt/vol) PurSil polymer solution in THF and dried for 8 h to remove any THF via evaporation. To fabricate the NO-releasing osmium-mediated glucose sensor (Fig. 5.1b), a Pt-Ir wire insulated with a Teflon coating was inserted into a commercial silicone tubing (Artec) which was previously impregnated with the NO donor, S-nitroso-N-acetylpenicillamine (SNAP), via a solvent swelling method using 125 mg/ml SNAP in THF. Before insertion, the Teflon coating was coated with a layer of copper nanoparticles (Cu-NPs, 5 wt % and 40-60 nm) in Carbosil (2080A, DSM) in THF solution. Then, at the distal tip of the device, sequential layers of glucose oxidase, PVI-Os (III) and PEGDE, then Nafion and 2 % PurSil in THF were applied with drying in between each layer (see Fig 5.1b). The analogous control sensors were prepared by only inserting the osmium-mediated glucose sensor (with the sensing area at the distal tip) into the silicone tubing, but without the SNAP and Cu-NP layers.

5.2.2 Sensor Performance

Figure 5.2 shows that the basic osmium-mediated glucose sensors (Fig. 5.1a) without any NO-releasing outer tubing exhibit stable amperometric responses to glucose in the range of 0-20 mM using an applied potential of only +200 mV vs. Ag/AgCl between the Pt/Ir electrode and the wrapped Ag/AgCl reference electrode. As also shown, this response is quite stable for at least 5 days, when the sensor is stored and tested in phosphate buffer solution (PBS, pH 7.4) at 37 °C, with excellent linearity ($R^2 = 0.9963$) and reasonably fast response times (3-5 min). The sensitivity
The normalized sensitivity of the basic osmium-mediated glucose sensors (n = 3) on day 1, 3, and 5 day are as follows: 117.4 % and 118.4 % on Day 3 and 5, respectively. These values were calculated by dividing the sensitivity on each day by that on day 1.

5.3 Results and Discussion

5.3.1 Effect of Nitric Oxide on Osmium-Mediated Chemistry

To determine whether nitric oxide can cause additional background current to glucose sensors based on hydrogen peroxide detection (+0.65 V vs. Ag/AgCl reference), but not to the sensors based on osmium-mediated chemistry (+0.2 V), both types of glucose sensors were prepared without any NO-releasing outer tubing, and were placed in a bulk PBS solution (pH 7.4) with 0 mM glucose in the absence (purged with N₂) and the presence of 300 ppm NO. Notably, the concentration of a saturated NO solution is reported to be 1.74±0.03 mM (equivalent to 52.2 ppm). As shown in Figure 5.3, when 300 ppm NO was introduced at the 10 min mark, the
osmium-mediated glucose sensor exhibits no additional background current while the sensor based on hydrogen peroxide detection exhibits a significant increase in current (~5 nA) (equivalent to 4.1 mM glucose based on prior calibration curve for that sensor) due to electrochemical oxidation of NO. This result confirms that the application of lower redox potential (+0.2 V) avoids the unnecessary additional background current from oxidation of NO at the inner electrode surface of sensing area.

To determine whether nitric oxide has any adverse effects on the electron-transfer process during glucose measurements, osmium-mediated glucose sensors prepared without any NO release outer tubing (Fig. 5.1a) were used to measure 14 mM glucose in bulk PBS solution (pH 7.4) while simultaneously purging the solution continuously with 300 ppm NO gas over a 25 h period. The osmium-mediated glucose sensor exhibits very stable and continuous measurement of 0 mM glucose (background current) between 0-3 h and 14 mM glucose between 3-25 h in both the absence (0-1 h) and presence (1-25 h) of 300 ppm NO (see Fig. 5.4). Furthermore, not only did NO fail to provide any additional background current at the applied potential of +200 mV (as
shown in Fig. 5.4 between 1-3 h), but the sensor also exhibited relatively stable and continuous measurement of the 14 mM glucose concentration in the presence of 300 ppm NO, more than the concentration of saturated NO in solution (52.2 ppm),\textsuperscript{27} for 22 h (<4 % change in current over this period). These measurements match the calibration curve (N\textsubscript{2}-purged solution) obtained prior to the experiment (inset in Fig. 5.4). As a result, the osmium-mediated glucose sensor exhibits continuous glucose sensing capability in the presence of highly concentrated levels of NO within the test solution.

\textbf{5.3.2 Nitric Oxide Flux Analysis}

As shown in Figure 5.5, the additional layer of Cu-NPs in Carbosil between the Teflon coating on the Pt working electrode and SNAP-impregnated silicone tubing increases the NO release flux. The Cu\textsuperscript{+} ions, produced from oxidation of Cu\textsuperscript{0} nanoparticles in the presence of moisture, catalyze the generation of NO from RSNOs such as SNAP.\textsuperscript{29-31} Indeed, the SNAP- impregnated silicone tubing without the Cu-NP coatings exhibited lower NO fluxes compared to the Cu-NP based devices. The NO fluxes from SNAP with Cu-NPs correspond to the physiological levels (0.5-4 × 10\textsuperscript{-10} mol·min\textsuperscript{-1}·cm\textsuperscript{-2}) of NO released by endothelial cells that line the walls of all blood vessels.\textsuperscript{32}
Nitric oxide released from the osmium-mediated glucose sensor was measured in real-time via chemiluminescence using a Sievers Nitric Oxide Analyzer (NOA) 280.

### 5.3.3 Detection of Copper Leaching

Detection of the copper ion leaching from the polymeric composites (e.g., Carbosil) is crucial for the preclinical testing of an implantable device, as diffusion of copper ions from the polymers will highly influence cell cytotoxicity. After 72 h of soaking in 8 mL of PBS (pH 7.4) at 37 °C to allow any potential copper ion leaching, the glucose sensors containing the Cu-NPs in the inner Carbosil layer were removed from the solution and a standard ICP-MS method was employed to monitor the degree of copper leaching from the sensors. The results of the detected copper leaching are shown in Table 5.1. Measured copper leachate from the sensors was well below cytotoxic concentrations for mammalian cells. Previous reports have indicated that cell viability stays at 100% until the concentration of copper reaches approximately 1000 ppb.\textsuperscript{33-35} The result demonstrates that the concentration of leached copper is insignificant and therefore should exhibit no adverse effect on sensor biocompatibility.

**Figure 5.5.** NO flux analysis of SNAP-based NO release glucose sensors prepared with and without Cu-NPs for 3 consecutive days (note: 1 flux unit = $1 \times 10^{-10}$ mol·min$^{-1}$·cm$^{-2}$). The error bars represent standard deviation for n = 3.
**Table 5.1.** Copper leachate concentration from Cu-NPs in Carbosil layer in osmium-mediated glucose sensor and relative leaching in PBS as compared to the original amount of copper present in each sensor

<table>
<thead>
<tr>
<th>sample</th>
<th>initial Cu-NPs (µg)</th>
<th>measured copper leachate in 8 mL PBS after 72 h (ppb)</th>
<th>% copper leaching after 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-NPs</td>
<td>19.05 ± 1.695</td>
<td>152.7 ± 4.967</td>
<td>6.45 ± 0.50</td>
</tr>
<tr>
<td>Cu-SNAP</td>
<td>18.25 ± 0.4490</td>
<td>74.59 ± 27.06</td>
<td>3.29 ± 1.25</td>
</tr>
</tbody>
</table>

5.3.4 Sensor Performance on Benchtop

As shown in Figure 5.6, although the NO-releasing glucose sensor exhibits slightly different glucose sensitivity than the control, this difference is not due to interference from NO, but is rather due to the fabrication process, as the amount of reagents (GOX, PVI-Os, and outer PU coating) deposited on sensing area directly dictates the current output for glucose measurements. Most importantly, continuous NO release from SNAP does not exhibit any negative effect on the osmium-mediated chemistry for glucose measurement over a 3-day period. The normalized sensitivity of NO-releasing and controls sensors \((n = 3)\) over 3 days are as follows: 109.1 and 86.4 % on Day 2 and 3 for NO-releasing sensors, respectively; 87.7 and 97.7 % on Day 2 and 3 for control sensors, respectively. The percentage was calculated from dividing the sensitivity on each

![Figure 5.6. Glucose calibrations (0-20 mM) with applied potential of 200 mV in PBS (pH 7.4, 37 °C) for 3 consecutive days. (a) NO-releasing glucose sensor; (b) control glucose sensor. (note:1 flux unit = 1 x10^-10 mol·min⁻¹·cm⁻²).](image-url)
In vitro continuous glucose measurement in heparinized whole porcine blood (female, 120 kg, provided by Extracorporeal Life Support Laboratory at University of Michigan) was conducted with NO-releasing and control osmium-mediated glucose sensors for 3 consecutive days. On each day, glucose values were back-calculated using the calibration obtained prior to the in vitro blood testing. To confirm the accuracy of the glucose sensors, a commercial blood glucometer (Accu-Chek® Nano, Roche Diabetes Care) was used every 10 min to measure (in triplicate) the whole blood glucose level. This value was compared to the value obtained from the sensors using the prior calibration data. NO release was monitored for the three days via collecting NOA data for one hour each day, prior to glucose calibration (0-20 mM) in PBS (pH 7.4, 37 °C) and the in vitro continuous glucose measurements in porcine blood. The sensors were
stored in PBS (pH 7.4) at 37 °C in the dark between measurements to ensure continuous NO release from SNAP.

As shown in Figure 5.7, the intermittent glucometer glucose measurement values in heparinized whole porcine blood are very comparable to the continuous glucose measurements obtained by the osmium-mediated glucose sensors with the pre-calibration made in PBS on each day. The discrepancies in glucose value between NO-releasing glucose sensor and glucometer observed at the 2 min mark may be have resulted from glucometer test strips having the standard accuracy of ±15% for measured glucose concentration. Regardless, it is clear that during 3 days of continuous NO release from the sensors, at or above physiological levels, no negative effects on the osmium-mediated wired enzyme electrode chemistry are observed.

5.3.5. Improving Sensors to Reduce Oxygen Sensitivity

The NO release osmium-mediated glucose sensors were further optimized to avoid interference from oxygen by switching the glucose-oxidizing enzyme from glucose oxidase (GOX) to glucose dehydrogenase (GDH). The GDH utilizes flavin adenine dinucleotide (FAD) as a cofactor within the structure of the enzyme. Indeed, GOX can utilize both PVI-Os and oxygen as the electron acceptor which may generate unintended errors due to variations in the oxygen concentration/saturation of the blood (e.g., higher oxygen levels reduce the signal). However, FAD-GDHs are unable to utilize oxygen as an electron acceptor, despite possessing significant structural similarities with GOX.36 As shown in Figure 5.8 (left), osmium-mediated glucose sensors that now utilize FAD-GDH as the glucose-oxidizing enzyme exhibit excellent linear sensitivity to glucose and selectivity over possible interference species such as 0.5 mM ascorbic
acid (AA) and 0.2 mM acetaminophen (AC). And the current response to 20 mM glucose is not altered before and after purging the sample solution with nitrogen gas at the 84 min mark. This suggests that the PVI-Os is the only electron acceptor utilized by FAD-GDH with an applied potential of +200 mV vs. Ag/AgCl. Furthermore, the new sensor design with FAD-GDH exhibited relatively stable and continuous measurement of the 20 mM glucose in the presence of gas phase NO at 100 ppm nitrogen, more than the concentration of saturated NO in solution (52.2 ppm),\textsuperscript{27} for 24 h (<4 % change in current over this period). As a result, the new osmium-mediated glucose sensor utilizing FAD-GDH as glucose-oxidizing enzyme exhibits continuous glucose sensing capability in the presence of highly concentrated levels of NO within the test solution.

5.3.6 Sensor Performance in Vivo

These new FAD-GDH based NO release glucose sensors were employed in a porcine model (\(\sim 50\) kg, \(n = 2\) pigs) to determine their accuracy in monitoring blood glucose levels continuously \textit{in vivo}. The sensors were placed in the femoral and carotid arteries of a pig under anesthesia, without any

\textbf{Figure 5.8.} (Left) Glucose calibrations (0-20 mM) and obtained by new osmium-mediated glucose sensors (\(n = 4\)) that utilize FAD-GDH as glucose-oxidizing enzyme with applied potential of 200 mV in PBS (pH 7.4, 37 °C). (Right) Continuous measurement of 20 mM glucose (0-24 h) using the new osmium-mediated glucose sensors (\(n = 3\)) with applied potential of 200 mV in the presence of gas phase NO at 100 ppm in nitrogen. The electronic noise recorded after 84-min mark is from bubbling of N\textsubscript{2} into the solution.
anti-coagulant administration, via an open cut-down allowing for continuous blood flow past the sensors for 19 h. The \textit{in vivo} glucose level was measured continuously and changed periodically with 20 g of dextrose injections (ca. every 3 h). Arterial blood was drawn at 10 min, 20, 30, 60, 90, 120 and 180 min mark after every dextrose injection to assess the accuracy of the glucose

Figure 5.9. Performance of electrochemical NO-releasing osmium-mediated glucose sensors implanted in pig arteries for 19 h at +200 mV vs. Ag/AgCl. \textit{In vivo} comparison of intermittent glucose values obtained from a benchtop blood gas analyzer (red circles) and the converted current values measured by (a) two control sensors and (b) two NO-releasing sensors 1-point calibration was applied to data obtained by NO-releasing sensors at 10 h time point. (c) Representative photo illustrating the degree of clot formation on the surface of controls and the NO-releasing sensors after being explanted. (d) Average clot formation area on controls vs. NO-releasing sensors (n = 3, p < 0.05).
values provided by the implanted sensors. At the end of the in vivo experiments, the sensors with the blood vessels intact were explanted after systemic heparinization to prevent necrotic thrombosis during vessel harvesting; digital pictures were taken, and the red pixels were counted using ImageJ software to quantify clot area.\textsuperscript{37-38} The in vivo data from the sensors were recorded every second and averaged every 30 s to reduce the electronic noise as well as the size of the data set. A 1-point calibration was applied at the 10 h-point to further correct for the slight change of inherent sensitivity of glucose sensors with time (see Figure 5.6). As shown in Figure 5.9 (a) and (b), the NO-releasing sensors exhibited more reliable continuous glucose measurement for the entire 19 h in vivo experiments while the control sensors exhibited a significant deviation at almost every time point after 6 h of implantation. This was likely due to the formation of the large thrombus formation on the control sensor (see Fig. 5.9c, as an example). Thrombus formation around the sensing area can prevent direct contact with blood for glucose measurement. Overall, the NO releasing sensors induced less clot formation than the control sensors as quantitated by imaging the surface of the sensors following explantation from the pig after 19 h (see Fig. 5.9d, n = 3 for each type of sensor). Similar analytical results for most of the control and NO release sensors were obtained in the second animal study that was conducted with these new NO release in vivo glucose sensors (data not shown).

5.4 Conclusion

In summary, it has been shown that in vivo type amperometric glucose sensors prepared with the Os(III/II)-bipyridine-PVI complex maintain continuous analytical functionality and measurement accuracy in the presence of nitric oxide. Further, there is no evidence that NO can be oxidized at the underlying Pt/Ir working electrode at the applied potential of +200 mV vs. Ag/AgCl used to
record the current response from these devices. In addition, there does not appear to be any direct interaction between the NO and the Os(III/II) redox center that inhibits electron transfer from the glucose oxidase enzyme and this redox site. Similar behavior was observed when glucose oxidase or FAD-GDH was used as the wire enzyme of the sensors. The FAD-GDH system was found to not exhibit any oxygen sensitivity making it more useful for continuous in vivo glucose sensing. Preliminary data on the performance of these sensors in a pig model over a 19 h period are promising in terms of glucose sensing accuracy. While the ultimate focus of this research was to prepare useful IV glucose sensors, incorporation of NO release also has the potential to improve the performance (reduce drift, require less frequent calibrations) of similar wired enzyme electrodes employed for subcutaneous glucose sensing. This is because it is well known that NO release can also greatly reduce the foreign body inflammatory response that occurs for devices placed in the subcutaneous space.39-40

5.5 References

CHAPTER 6
Conclusions and Future Directions

6.1 Conclusions
Diabetes mellitus has become a leading cause of death worldwide. To date, although there is no ultimate cure to prevent or reverse diabetes, only targeted administration of appropriate medication and use of various blood glucose monitoring systems to enhance treatment efficiency, can help alleviate the symptoms and diminish the complications of the condition. This dissertation aimed to address and improve both a non-invasive and a continuous in vivo approach to glucose monitoring and thereby improve the measurement technology, potential patience compliance, and ultimately, patient outcome.

In Chapter 2, test-selected lots of commercially available ACCU-CHEK® Aviva Plus glucometer strips manufactured by Roche were demonstrated to exhibit the sensitivity and selectivity necessary to measure low levels of glucose in human tears using sample volumes of <1 µL. Preliminary measurements of tear glucose using these strips in non-diabetic volunteers match well with those reported by Asher\(^1\) with research laboratory LC-MS instrumentation.
In Chapter 3, it was found that the ability of Roche Accu-Chek test strips to be used to measure μM levels of glucose without substantial interference from other redox active species is specifically due to the unique combination of using PQQ-GDH as the active enzyme in combination with a nitrosoaniline derivative as an electron transfer mediator. The low $K_M$ value of this enzyme, coupled with the inability of uric acid and acetaminophen to reduce the nitrosoaniline mediator and yield electrochemical response in a biamperometric measurement mode, appear to be the key elements that provide the excellent LOQ value and selectivity of these strips.

In Chapter 4, the ACCU-CHEK Aviva glucometer test strips were used to measure glucose levels in tear fluids from $n=4$ human subject with Type 2 diabetes over 1.5 h period while also concurrently measuring the blood glucose values. Measurements of blood and tear (in both eyes) glucose levels were made immediately before having the subjects consumed a tube of sugar jelly, and then for 1.5 h thereafter at 30 min intervals. A moderate correlation between tear and blood glucose levels was found for these diabetic patients, suggesting that measurement of tear glucose is a potential noninvasive supplement method for monitoring blood glucose levels. However, it appears that the ratio of glucose levels in tear fluid vs. blood is variable from patient to patient, and hence this approach may require calibration of this ratio for each patient.

In Chapter 5, *in vivo* type amperometric glucose sensors prepared with the Os(III/II)-bipyridine-PVI complex were shown to maintain continuous analytical functionality and measurement accuracy in the presence of nitric oxide release. Further, there is no evidence that NO is oxidized at the underlying Pt/Ir working electrode at the applied operational potential of $+200 \text{ mV}$ vs.
Ag/AgCl reference electrode used to record the current response from these devices. In addition, there does not appear to be any direct interaction between the NO and the Os(III/II) redox center that inhibits electron transfer from the glucose oxidase enzyme and this redox site. By using FAD-glucose dehydrogenase (FAD-GDH) in place of GOX as the glucose selective enzyme, there was no interference from variable levels of oxygen in the sample phase, and this would be quite advantageous for use of such sensors for continuous IV monitoring of glucose in patients within ICU or CC units within hospitals.

Overall, this thesis work demonstrated significant potential improvements in both non-invasive and continuous glucose monitoring systems. The tear glucose measurement approach requires more extensive clinical studies to definitively establish the true correlation between tear and blood glucose levels, and how variable the ratio of these values are from patient to patient. The continuous glucose sensing devices using osmium-mediated chemistry and SNAP-doped silicone tubing aim to bridge the divide between the basic research on continuous monitoring glucose electrochemical sensors and a more clinically viable commercial product. Indeed, the use of wired FAD-GDH to ultimately develop an improved NO releasing intravascular glucose sensor that can provide more accurate, reproducible, and continuous real-time glucose measurements for critically ill hospital patients without risk of clotting (or infection; owing to NO’s potent antimicrobial activity) seems like a very promising new approach.
6.2 Future Directions

6.2.1 Tear Fluid Acquisition Method for Tear Glucose Measurement

The future clinical potential for diabetes management of tear glucose testing with glucometer strips requires thorough evaluation in human subjects, including responses to the full range of blood glucose levels during hyperglycemia and, especially, hypoglycemia. The practicality of self-acquisition of tear sample must also be addressed for safety and efficacy before such a method could be clinically employed.

Collecting large volumes of tear fluid (>1 µL) can cause a significant amount of irritation and induce stress on the ocular surface. Leakage of intercellular fluid from damaged conjunctiva can occur and consequently increase measured tear glucose levels. Elevated tear glucose levels can be prolonged for up to 30 minutes, further skewing the correlation with blood glucose. Therefore, to accurately detect tear glucose concentration, it is important to minimize the collection volume to sub-microliters and ensure no eye irritation occurs throughout the process.

Tear production via non-stimulation is the most reliable method for tear fluid collection which typically utilizes capillary tubes to collect ~1 µL of tear fluid by gently touching the tear film on ocular surface. However, given the safety concern of holding a small tube on a subject’s eye, such tests would have to be done by trained clinical personnel (as performed in Chapter 4) adding additional challenge to collection methods. Moderate correlation between tear and blood glucose levels was observed but variation within individuals and uncontrolled irritation still is challenging
the viability of using tear glucose measurement for optimal glycemic control among diabetic patients.

Requirements for an operational tear glucose sensor device must include the ability to collect a low volume of fluid and cause no irritation to the eye. Indeed, Labelle et al. proposed a prototypical fluid capture device that would utilize a soft foam plug to collect a small amount of tear fluid (see Figure 6.1). Their potential design aims to provide a supplementary method to blood glucose monitoring system that is non-invasive, rapid, and safe, which may ultimately increase patient compliance. Testing of such a device in conjunction with the glucometer strip measurement of tear glucose would be a logical next step for future research in this area.

6.2.2 Development of Insulin Delivery Cannula with Built-In Osmium-mediated Glucose Sensor

A combination of continuous glucose monitoring system with continuous subcutaneous insulin infusion therapy has led to significant reduction in hemoglobin A1c (HbA1c) in subjects with type 1 diabetes. Therefore, a single device that delivers both insulin infusion and continuous subcutaneous glucose monitoring would likely be an optimal strategy to manage changing glucose levels of type 1 diabetic patients, considering that insulin infusion therapy causes insignificant
perturbation to subcutaneous glucose levels at sites adjacent (e.g., 0.9 cm) to insulin infusion location.

Indeed, Ward et al.⁶ evaluated the performance of both H₂O₂-detection and redox mediator glucose sensors in anesthetized nondiabetic swine for potential interferences in insulin formulations and reported that phenolic preservatives present in insulin formulations can cause significant interference in glucose sensing by being oxidized at high redox potential (+0.6-0.65 V) used by H₂O₂-detection method. However, this problem can be avoided by using a redox mediator chemistry, such as osmium-mediated chemistry, that requires much lower redox potential of +0.175 V vs. Ag/AgCl (as shown in Fig. 6.2). Therefore, the research reported in Chapter 5 of

**Figure 6.2.** Glucose-sensing cannula results obtained in anesthetized nondiabetic swine using both H₂O₂-detection electrode (upper figure) and redox mediator sensor (lower figure). Arrows at 0 min time-marks in both upper and lower figures represent the timing of lispro insulin delivery. Note significantly high spurious current response to the insulin formulation in upper figure (from ref. 5).
this thesis on osmium-mediated continuous glucose sensors that uses a redox potential of +0.15 V, would likely be a perfect candidate to be coupled with insulin infusion therapy for an ultimate bifunctional infusion cannula/glucose sensor system.

Indeed, ongoing research by Dr. Xuewei Wang in this lab has focused on developing such insulin delivery cannula system integrated with the osmium-mediated glucose sensor that is immune to phenolic preservatives present in insulin formulations. Furthermore, nitric oxide, a potent vasodilatory, anti-inflammatory, antimicrobial/bactericidal, and pro-angiogenesis agent can be utilized to mitigate foreign body response of such a subcutaneous device to have an extended lifetime (e.g., 2 to 4 weeks). In his preliminary work (Fig. 6.3), the glucose sensors based on H$_2$O$_2$-detection exhibits stable sensitivity to glucose levels between 0 to 20 mM in phosphate buffer solution and releases nitric oxide > 0.5 x 10$^{-10}$ mol-cm$^{-2}$-min$^{-1}$ flux from SNAP-impregnated cannula for 18 days. This cannula system is now in the process of being coupled with osmium-mediated glucose sensor, instead of H$_2$O$_2$-detection method, to evaluate its performance in the presence of phenolic preservatives in insulin formulations.

![Figure 6.3. Calibration curves obtained using glucose sensors based on H$_2$O$_2$-detection (left) and NO fluxes (right) of the integrated device in a 18-day experiment in phosphate buffer solution.](image-url)
6.2.3 Development of NO-releasing 3rd Generation Glucose Sensor

3rd Generation biosensors employ direct electron transfer between the electrode and the active site of the enzymes, with a low redox potential of the enzyme itself, eliminating the need of toxic and artificial electron mediators, thereby avoiding errors caused by different concentration of oxygen.
in blood. Unfortunately, only a few enzymes are able for direct electron transfer between their active sites to the electrode. Cellobiose dehydrogenase (CDH) has been received great attention as the active enzyme for 3rd generation biosensors due to its ability to directly transfer electrons with the electrode. Therefore, CDH would be a great candidate to investigate for the compatibility with nitric oxide release, to improve biocompatibility of a potential intravascular 3rd generation biosensors.

Indeed Bollella et al. recently reported that CDH and a novel glassy carbon electrode modified with direction electrodeposition of gold nanoparticles are capable of creating efficient direct electron transfer to be useful as a glucose biosensor. As shown in Figure 6.4, the biosensors are able to yield a low detection limit toward glucose of 6.2 µM, a great linearity (R² = 0.995) in the wide range between 0.02 and 30 mM with an applied potential of +0.25 V vs. Ag/AgCl, which could be advantageous in selectivity over potential interferents such as ascorbic acid, uric acid and acetaminophen. Therefore, it would be interesting to investigate the compatibility of CDH with NO release for improving the biocompatibility of potentially implantable glucose biosensors.

6.3 References

