POINT-OF-CARE SENSORS FOR DIABETES AND MYOCARDIAL ISCHEMIA

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Cardiovascular disease is the leading cause of death in the United States, and over 20 million people in this country currently have diabetes. We have developed new analytical methods that have the potential to improve the monitoring and detection of these diseases. Our work enables point-of-care sensing for biochemical markers of these diseases, advancing the non-invasive monitoring of diabetes and the rapid detection of myocardial ischemia.

Close monitoring of blood glucose concentration is critical in the management of diabetes. A recent approach to non-invasive monitoring is to measure glucose concentration in tear fluid. However, the relationship between tear and blood glucose concentration is poorly understood. We have developed an analytical method for determining tear glucose concentration, and have used it to help better define the correlation between tear and blood glucose in both fasting and non-fasting human subjects. We also reviewed the tear glucose literature from the last 80 years, and have used our method to help explain some of the discrepancies reported. This work will aid in developing contact lens-based sensors for tear glucose, and may help to decrease or eliminate finger-stick testing.

The definitive detection of myocardial ischemia is difficult. While some patients are sent home with an undetected heart attack, many are unnecessarily admitted to the hospital, costing the medical system ~$12 billion a year. The Cobalt Binding Assay is a test that has shown promise in ruling out myocardial ischemia. However it is limited by the need to access a central

clinical chemistry laboratory. We have developed a Polymerized Crystalline Colloidal Array (PCCA) sensor that could be used at the bedside to rapidly detect myocardial ischemia. This sensor uses photonic crystal and hydrogel technology to actuate a color change in response to metal cation binding affinity changes indicative of ischemia. Specifically, the sensors monitor a decrease in the binding affinity of human plasma for metals such as Ni^{2+} and Co^{2+}, which has been shown to correlate with recent ischemia. We have also developed improved methods for fabricating and testing the PCCA sensors that are applicable to implementing point-of-care sensors for a wide variety of analytes.

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PREFACE

I would like to thank, first of all, my research advisor, Professor Sanford Asher, for his mentorship and support. I have had remarkable opportunities to grow as a scientist while working in his lab, and he has provided a fertile environment for me to develop my critical thinking skills. I have learned much from his approach to managing a large, successful laboratory, and I am confident my experiences here will serve me well in the future.

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1.0 INTRODUCTION

1.1 POINT-OF-CARE SENSING WITH POLYMERIZED CRYSTALLINE COLLOIDAL ARRAYS

Cardiovascular disease is the leading cause of death in the United States, and over 20 million people in this country currently have diabetes. We have developed new analytical methods that have the potential to improve the monitoring and detection of these diseases. Our work enables point-of-care sensing for biochemical markers of these diseases, advancing the non-invasive monitoring of diabetes and the rapid detection of myocardial ischemia. A common thread in these studies is the development of Polymerized Crystalline Colloidal Arrays (PCCA) sensors for point-of-care sensing applications.

PCCA sensors are comprised of a hydrogel matrix that contains a ordered array of monodisperse colloidal nanoparticles.[1] In aqueous solutions with low ionic strength, a Crystalline Colloidal Array (CCA) forms when highly charged polystyrene spheres self-assemble into a body centered cubic or face centered cubic lattice.[2] Particle diameter and concentration can be selected so that the CCA Bragg-diffracts light in the visible range. A PCCA is formed when a hydrogel, such as polyacrylamide, is polymerized around the CCA. Swelling or shrinking of the hydrogel causes the lattice plane spacing to change, and the wavelength of diffracted light is a function of hydrogel volume. PCCA sensors are made by modifying the

hydrogel so that a volume change occurs in response to a specific analyte. In the high ionic strength of most biological fluids, a straightforward way to actuate a change in hydrogel volume is to cause crosslinks in the hydrogel to form or break. The fabrication of PCCA sensors is discussed in greater detail in Chapters 5 and 6.

A wide variety of PCCA sensors for clinically relevant analytes have been demonstrated including ammonia,[3] creatinine,[4] lead,[5] and glucose.[6] One of the promising point-of-care applications for PCCA sensors has been for the non-invasive monitoring of glucose in the tear fluid. PCCA sensors for glucose could easily be incorporated into a contact lens.[7] The contact lens could be worn by diabetic patients, who could simply look in a mirror to determine their blood glucose levels. The success of this approach depends on the relationship between tear and blood glucose concentrations. This relationship, however, is poorly defined, and there are several contradictory reports of tear glucose concentration in the literature.

We undertook several studies of the glucose concentration in tears in order to help define the basal tear glucose concentration, determine the relationship between blood and tear glucose concentrations, and advance the use of PCCA glucose sensors for the non-invasive monitoring of diabetes. The first study (Chapter 2) describes the development of an electrospray mass spectrometry method to measure low concentrations of glucose (<100 μM) in small volumes (~1μL). The second study (Chapter 3) describes the use of this method in a clinical study that measured tear glucose in fasting, non-diabetic subjects. Additional studies of healthy and diabetic subjects undergoing glucose tolerance tests were then combined with a broad review of the tear glucose literature to present an overview of the current understanding of tear glucose regulation (Chapter 4).

We also present the development of a new application for PCCA sensing (Chapter 5), which is the rapid detection of myocardial ischemia. The rapid and definitive detection of myocardial ischemia is difficult; while some patients are sent home with an undetected heart attack, many are unnecessarily admitted to the hospital. The Cobalt Binding Assay is a clinical chemistry test that detects a change in the binding affinity of human serum for cobalt. A decreased binding affinity of human serum for cobalt has been shown to correlate with recent myocardial ischemia in several clinical studies. While this assay was developed to monitor a change in cobalt binding, the ischemia-linked change in metal binding affinity appears to be general for other divalent transition metal cations, including Ni^{2+} and Cu^{2+}. The precise mechanisms responsible for a change in the metal binding affinity of human serum after ischemia have not been fully explained, but apparently relate to a change in the redox potential of the blood.

The Asher group has previously demonstrated PCCA sensors for metal cations such as Co^{2+}, Ni^{2+}, and Cu^{2+} in acidic solutions.[8] We have developed these PCCA sensors for the detection of Ni^{2+} in the presence of human plasma. This enables the rapid detection of changes in the affinity of human serum for Ni^{2+}. While the Cobalt Binding Assay requires access to a central clinical chemistry laboratory and involves intensive sample processing, our PCCA sensors could be used in a point of care setting to rapidly detect myocardial ischemia.

The development of these PCCA sensors for the measurement of Ni^{2+} in human plasma also led to general improvements of PCCA fabrication methods. One of the challenges of using PCCA sensors is that they must be immobilized against a flat surface to insure that diffraction is monitored along the normal direction. We report on the development of methods to fabricate uniform PCCA sensors that are attached to a gel support film (Chapter 6). Once attached to a

support film, it is much easier to manipulate the sensors and to immobilize them for sensing experiments.

1.2 TEAR GLUCOSE ANALYSIS

Development of an electrospray ionization mass spectrometry method for the analysis of tear glucose concentration was done in collaboration with Christopher Taormina and reported in the Journal of the American Society for Mass Spectrometry.[9] My contributions were collecting the tear samples and helping to optimize and refine the analysis method.

Briefly, we developed a mass spectrometry-based method for the accurate determination of glucose concentration in tear fluid. We use 1μL capillaries to collect tear fluid from the tear fluid meniscus of human subjects. This collection method was specifically chosen to avoid irritation of the eye and the stimulation of reflex tearing. We used liquid-chromatography electrospray ionization mass spectrometry with D-glucose-6,6-d2 as an internal standard. Repeated measurement of a pooled tear sample induced by onion vapors showed that the measurement of tear glucose with this method is precise (4% relative standard deviation). A recovery experiment showed complete recovery of added glucose (94-111% recovery range). The tear glucose concentration in a fasting, non-diabetic subject ranged between 13 and 51 μmol/L and the tear glucose concentration in onion-stimulated tear from a different non-diabetic subject ranged between 211 and 256 μmol/L.

1.3 CLINICAL STUDY OF FASTING, NON-DIABETIC SUBJECTS

We reported a clinical study of basal tear glucose concentration in fasting, non-diabetic subjects using the electrospray ionization mass spectrometry method in Clinical Chemistry.[10] This study was undertaken to help understand the discrepancies in tear glucose concentrations reported in the literature. We collected three 1μL tear samples from both eyes of 25 fasting volunteers. Eleven of the subjects wore contact lenses during tear sampling. We measured a median tear glucose concentration of 28 μmol/L with a range of 7-161 μmol/L. We found no significant difference between subject who wore contact lenses and those who did not, and observed a significant correlation between tear and blood glucose concentrations. We also found that the standard deviation of tear glucose concentration increases linearly with the average tear glucose concentration for individual subjects. The tear glucose concentrations we measured were some of the lowest ever reported in the literature, most likely because our sampling methods avoided conjunctival irritation and tear stimulation.

1.4 REVIEW OF TEAR GLUCOSE SENSING LITERATURE

In order to make sense of the discrepancies in the tear glucose literature, we prepared an extensive review of tear glucose physiology and analysis. This review covers the present understanding of the physiology of glucose transport in tears, the regulation of the aqueous tear fraction, and studies of tear glucose concentration over the last 80 years. The various tear collection methods employed greatly influence the measured tear glucose concentrations. Studies that involve mechanical irritation of the conjunctiva during sampling measure the highest

tear glucose concentrations, while studies that avoid tear stimulation measure the lowest concentrations. This review also covers attempts to monitor tear glucose concentration *in situ* by using contact lens-based sensing devices. We also present new observations of tear glucose concentration using our tear collection method purposely designed to avoid tear stimulation. These studies indicate the importance of the sampling method in determining tear glucose concentrations. On the basis of these results, we discuss the future of *in vivo* tear glucose sensing, and outline the studies needed to resolve the remaining questions about the relationship between tear and blood glucose concentrations.

1.5 MYOCARDIAL ISCHEMIA

We developed PCCA sensors Ni^{2+} in order to monitor changes in the affinity of human plasma for Ni^{2+} and enable the rapid, point-of-care detection of myocardial ischemia. We submitted this work for peer review to Clinica Chimica Acta. We fabricated PCCA sensors for Ni^{2+} using 5-amino-8-hydroxyquinoline as a binding agent. Sensors were tested in aqueous solutions and in diluted human plasma. Peak diffracted wavelength of the sensors was monitored by reflectance spectrometry and correlated with Ni^{2+} concentration. The PCCA sensors showed a linear response to aqueous Ni^{2+} concentrations between 0.2 and 1.0 mmol/L, and can detect changes in Ni^{2+} concentration as small as 150 μmol/L. The sensors respond at physiological pH and can be reversibly dehydrated. The sensors detected a decrease in free Ni^{2+} of 1.0 mmol per L of plasma added. These sensors could be used to detect a decrease in the Ni^{2+} affinity of plasma proteins which may indicate recent myocardial ischemia

1.6 FABRICATION OF UNIFORM PCCA SENSORS

We have also developed improved methods for fabricating and testing the PCCA sensors that are applicable to implementing point-of-care sensors for a wide variety of analytes. We found that degassing the colloidal suspension before polymerization aids in the attachment of PCCA sensors to gel support films. When one side of a polymerization cell is exposed to the atmosphere during photopolymerization, a rainbow-colored PCCA results. Uniform PCCA sensors attached to gel support films can be fabricated by carefully excluding oxygen from the polymerization cell.

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2.0 ANALYSIS OF TEAR GLUCOSE CONCENTRATION WITH ELECTROSPRAY IONIZATION MASS SPECTROMETRY

Taormina CR, Baca JT, Finegold DN, Asher SA, Grabowski JJ. J Am Soc Mass Spectrom 2007;18:332–6.

2.1 INTRODUCTION

In the United States, 9.6% of the population over 20 years of age and 20.9% of the population over 60 years of age have Diabetes Mellitus.[1] The Diabetes Control and Complications Trial has shown that close monitoring and tight control of blood glucose concentration is critical in preventing the complications of Diabetes.[2] Current self-monitoring methods rely on invasive finger sticks to directly measure blood glucose levels to provide the critical information required to achieve glycemic control. A number of non-invasive approaches to monitoring blood glucose concentrations are being pursued; however, none have been successfully developed to the point where they have gained widespread clinical acceptance.[3] There has been significant recent activity in exploring non-invasive monitoring by using tear fluid glucose as an indicator for blood glucose.[4-6] Such an approach will be effective only if the glucose concentrations in tear fluid are a reliable surrogate for blood glucose concentrations.

A survey of the literature over the last 70 years on tear glucose determinations indicates significant disagreement in measured tear glucose concentrations and on the relationship of tear fluid glucose to blood glucose concentrations. Daum and Hill[7] noted in their 1982 review of the human tear glucose literature that reported values of tear glucose in normal individuals range between 0 and 3600 μM with the median values between 110 and 280 μM. More recently, a study of six non-diabetic adults found glucose concentrations in tear fluid ranging between 128 and 166 μM with an average of 139 μM.[8]

The differences in tear glucose concentrations between these reports are likely due to the use of different tear fluid collection methods. Van Haeringen and Glasius specifically addressed the dependence of tear fluid glucose on the method of collection.[9] They used a glucose dehydrogenase method to analyze chemically stimulated tears collected with a capillary and mechanically stimulated tear fluid collected by filter paper. Van Haeringen and Glasius found higher glucose concentrations in the tear fluid collected by the filter paper and concluded that the increase is due to the mechanical stimulation of the corneal and conjunctival epithelium.[9] Other studies reported similar findings in experiments on rabbits[10] and in subjects that were tested immediately after swimming.[7] Therefore, it is apparent that to determine a physiologically-relevant, baseline tear glucose concentration, tear fluid must be acquired with minimal tear stimulation and eye irritation.

Several previous studies used mass spectrometry to analyze tears for constituents other than glucose. Fung *et al.*[11] characterized the protein profile in the human tear fluid with MALDI-ToF and electrospray ionization mass spectrometry. Ham *et al.*[12] identified the lipid profile in tear fluid and reported on their variations between normal and dry eyes. There have been many reports of glucose measurement by mass spectrometry on bodily fluids other than

tears. The majority of these studies use gas chromatography and an electron ionization source.[13-16] With gas chromatography, glucose typically needs to be derivatized to make it more volatile. Other studies used liquid chromatography to bypass the volatility issue.[17,18] Sodium,[19] lithium,[20,21] cesium,[17] and chloride[22] ions have been used with electrospray ionization to form the cationic adduct of glucose from aqueous solutions.

We discuss here a new, robust method of determining physiological tear glucose concentrations by using electrospray ionization mass spectrometry (ESI-MS). This method enables reliable studies of tear glucose concentrations in tear fluid. We use a tear fluid sampling method that is minimally irritating to the eye to collect 1μL tear samples. We validate the ESI-MS method by using glucose standard solutions, artificial tear fluid, and large volumes of stimulated tears. We determine tear glucose concentration in a non-diabetic, fasting adult.

2.2 EXPERIMENTAL

All tear samples were collected and transferred with 1 μL "Microcaps" micro-capillaries (Drummond Scientific Company, Broomall, USA). The tear film meniscus on the bottom eyelid was visualized using a slit lamp ophthalmic microscope (American Optics, Burlington, Canada). The micro capillary was gently touched to this tear fluid meniscus and the tear fluid was drawn in by capillary action. During half the collections, there was inadequate tear fluid to immediately fill the capillary with one touch. In these cases, the subject was asked to blink in order to redistribute the tear fluid and the capillary was again touched to the meniscus. This process was repeated until the capillary was completely filled. A few samples required as many as five repetitions of this process. Care was taken throughout tear fluid collection to avoid stimulation

of reflex tearing. The aqueous glucose standards were collected and transferred with the same process as a tear sample; the capillary was touched to the solution surface and was filled by capillary action. For studies involving larger volumes of stimulated tears, the subject chopped onions until tearing was induced. These tears were collected by capillary action with glass Pasteur pipettes (2 mL) and pooled for each subject in order to analyze a homogeneous sample.

Immediately after collection, the tear sample was transferred to a glass vial (Agilent, Palo Alto, USA) with a glass insert (Restek, Bellafonte, USA) containing 10 μL of acetonitrile (Mallinckrodt Baker, Inc., Phillipsburg, USA) and 10 μL of an aqueous stock solution of isotopically labeled glucose (D-glucose-6,6-d_2, 99% labeled, Cambridge Isotope Laboratories, Andover, USA) used as an internal standard. A typical value for the concentration of the D-glucose-6,6-d_2 stock solution was 27.5 μM, corrected for the 99% labeling (leading to a D-glucose-6,6-d_2 concentration of 13.1 μM in the sample injected into the mass spectrometer). The vials were then shaken and centrifuged for five minutes to ensure mixing and that the liquid was collected at the bottom of the vial. The samples were refrigerated at 5^{o}C until analyzed.

Glucose concentrations were measured using ESI-MS. Samples were analyzed by flow injection analysis on a HP 1000 LC-MS (Agilent, Palo Alto, USA). Initial studies used an autosampler connected directly to the mass spectrometer. However, our optimized method used a 2 μm filter and a Nova-Pak C_{18} 3.9 x 150 mm column (Waters, Milford, USA) placed in-between the autosampler and the mass spectrometer. In both methods, the mobile phase consisted of acetonitrile and water with 0.1% formic acid (Mallinckrodt Baker, Inc., Phillipsburg, USA) with a constant flow rate of 0.2 mL/min. The ratio of water to acetonitrile was 1:1 in the absence of the column and 17:3 with the column. The ion chromatograms of sodiated glucose isotopes (*m/z* 203 for natural glucose and *m/z* 205 for D-glucose-6,6-d_2) were

selected from the total ion chromatogram using the ChemStation software (Rev. A.06.03 [509]). These selected ion chromatograms were integrated to provide peak areas for the two glucose signals. During integration, the software option "baseline hold" was used. The peak areas of *m/z* 203 and 205 were used to determine the glucose-to-D-glucose-6,6-d_2 ratio. When calculating the peak area ratio, we took into account the 1.3% *m*+2 natural isotopic contribution of Na^+(glucose), *m/z* 203, to *m/z* of 205; 1.3% of the observed glucose peak area of *m/z* 203 was subtracted from the observed *m/*z 205 peak area to determine the area due solely to the internal standard. Each sample was injected three times at 6.5 μL per injection and the average glucose concentration recorded. All human samples were collected through protocols approved by the University of Pittsburgh Institutional Review Board.

2.3 RESULTS AND DISCUSSION

The cationization of glucose with H^+, Na^+, K^+, or Pb^{2+} was examined. An initial survey comparing negative ion to positive ion ESI suggested greater promise with positive ion methods. With the exception of sodium, millimolar concentrations of the acid or salt were needed to observe the glucose cation adducts as the base peak. The sodium-glucose adduct was present in all of the spectra and in the majority of them was the most abundant ion. Since sodiated glucose dominated even without the addition of sodium and since tear fluid contains ~160 mM of sodium,[23] our glucose analysis is based on the sodiated glucose ion. Further experiments were conducted to ascertain the solvent composition (water, acetonitrile, and methanol) that provided the maximum signal for sodiated glucose. Based on the results of these experiments, a 1:1 ratio of water to acetonitrile for the sample solvent was selected. For the experiments without the use

of the Nova-Pak C_{18} column, the 1:1 solvent ratio was also used for the mobile phase with the addition of 0.1% formic acid to the aqueous phase to produce more stable baseline ion currents. In our initial work, $^{13}C_6$-glucose was used as an internal standard but an unidentified peak at *m/z* 209 (which would interfere with the detection of sodiated $^{13}C_6$-glucose) was observed in the absence of the internal standard. Therefore, D-glucose-6,6-d_2 was a superior alternative since no signal was observed at *m/z* 205 in the absence of the internal standard.

With the optimal solvents and internal standard established, the effect of the biological matrix on the glucose signal was examined. The sodiated-glucose signal intensity observed for both artificial and real tear fluid was low compared to the background signal in our initial studies in which samples were directly infused into the mass spectrometer. Addition of a C_{18} column separated proteins, such as lysozyme, globulin, and albumin, and excess salts from the elution time of glucose. This in-line purification increased both the sensitivity and signal-to-noise ratio for sodiated-glucose. Analysis of the blank (a protein-free glucose-free artificial tear solution[4]) showed an average glucose concentration of 0.98 ± 0.56 μM. With the use of the column the statistical limit of detection was lowered to 3 μM. These improvements occurred in the presence of the column with the mobile phase optimized at a solvent ratio of 17:3 of water with 0.1% formic acid to acetonitrile which maximized the glucose signal and minimized overlapping peaks.

The precision of the micro-capillaries was measured by gravimetric means. Five individual capillaries were weighed, filled with water at 20°C, and weighed again. The average value for the water contained in the capillaries was 1.008 ± 0.013 mg (1.011 ± 0.013 μL at 20°C). Therefore, in all of our analysis of tears we assume a collection volume of 1.00 μL.

To test the linearity of the method, aqueous solutions with known glucose concentrations (0 – 200 μM) were prepared and analyzed. The linear regression of measured versus added glucose gave an equation of y = 0.99 x – 0.78 (R^2 = 0.98). The increase in the relative sodiated glucose signal *m/z* 203 can be easily seen when the mass spectra are normalized to the sodiated D-glucose-6,6-d_2 signal *m/z* 205 (Figure 2.1).

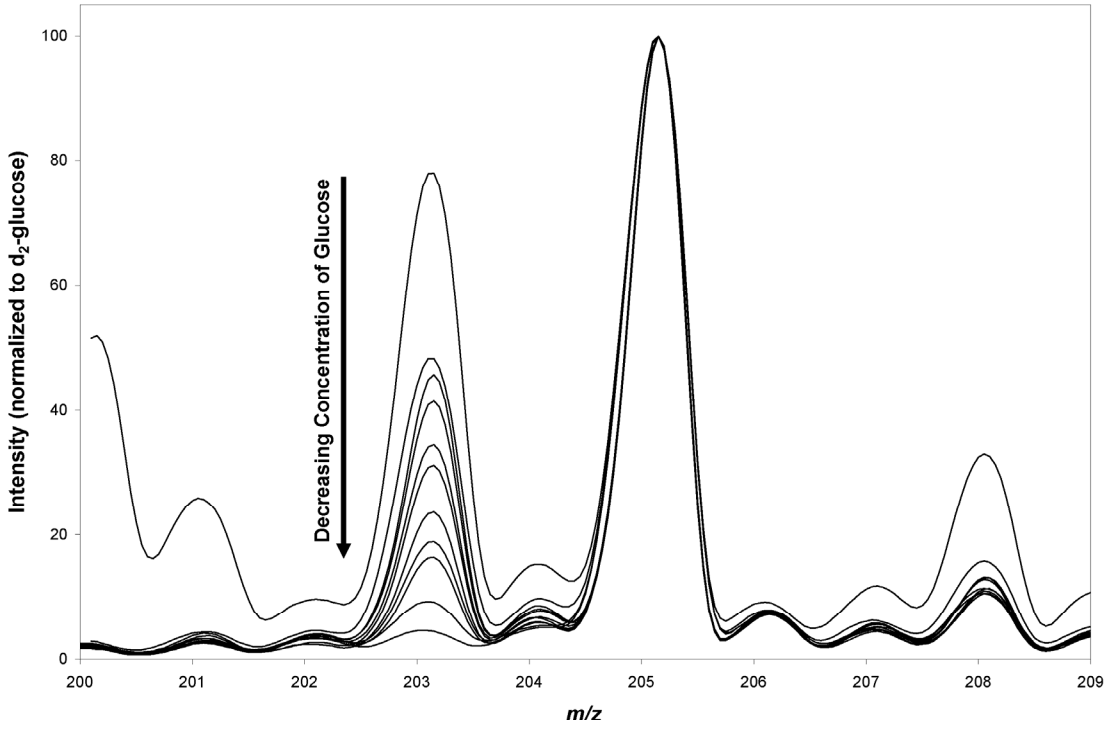

Figure 2.1. Eleven spectra of aqueous solutions of known glucose concentrations (0 – 200 μM) with intensities normalized to the internal standard D-glucose-6,6-d2 signal intensity at m/z 205. The linear regression of the experimental glucose concentration verses the known glucose concentration provided the best fit equation of y = 0.99 x – 0.78 (R^2 = 0.98).

The mass spectral measurement over time of the glucose concentration in the 1:1 ratio of water-to-acetonitrile was examined. A 200 μM glucose aqueous solution was prepared and

sampled multiple times. These vials were stored in a refrigerator and tested immediately, a few hours later, three days later, and one week later. The average value for all of the samples was 201 ± 15 μM with no increasing or decreasing trend over storage time (data not shown).

The reproducibility of the method was determined with the use of a pooled sample of onion-induced tears. Six samples were collected from the pool and analyzed. The average glucose concentration was 211 ± 8 μM in this stimulated tear sample (data not shown).

A recovery experiment was performed on another pooled sample of onion-induced tears. The tears were spiked with aqueous glucose solutions to increase the glucose concentration by 0, 107, 233, and 352 μM. Linear regression of measured versus added glucose gave an equation of $y = 0.99\ x + 256$ ($R^2 = 0.99$) (Figure 2.2). We also determined that the induced tear fluid contained 256 μM glucose.

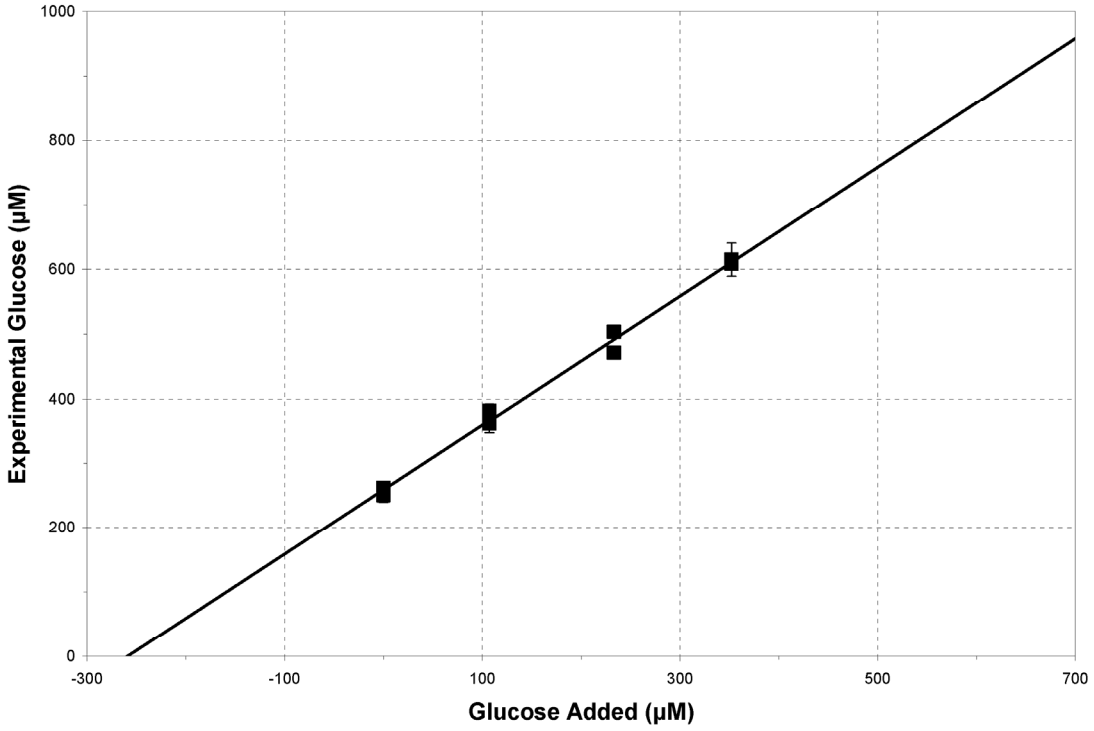

Figure 2.2. Duplicate samples of pooled, induced tears at four different concentrations of added glucose were analyzed. Error bars are plus/minus one standard deviation from the average value of each sample. Linear regression analysis gave an equation of y = 0.99 x + 256 (R^2 = 0.99) and a recovery range of 94% to 111% of the glucose spike.

The average relative standard deviation over the three 6.5 µL injections of the diluted tear samples studied with concentrations greater than 50 µM glucose was 4%. For the samples studied with concentrations less than 50 µM glucose, the average relative standard deviation of the three measurements was 15%. The FDA currently requires self-blood-glucose monitors to have an accuracy of within 15%. Within one standard deviation, the calibration curve is indistinguishable from y = x and the recovery experiment shows 100% recovery of added

glucose. We therefore feel confident using D-glucose-6,6-d_2 as the internal standard and the y = x calibration curve to determine tear glucose concentrations.

We performed two studies on a non-diabetic subject who fasted overnight. During both sessions, which were separated by one week, three samples were taken from each eye. Samples from the same eye were collected at ten minutes intervals. The tear glucose concentration from this subject during the two sessions ranged from 13 to 51 μM with an average of 32 μM (Table 2.1).

Table 2.1. Tear Glucose Concentration in μmol/L of a Non-diabetic Subject

	Session 1		Session 2	
Sample	Left Eye	Right Eye	Left Eye	Right Eye
1st	22	38	44	48
2nd	51	37	50	25
3rd	13	17	14	24

The concentration of tear glucose determined in this work for this non-diabetic subject is about four-fold less than the tear glucose concentrations recently reported.[8] Whereas previous studies used collection methods which either touched the eye or chemically stimulated tears,[7,9,10] we use glass micro-capillaries to collect a single microliter volume of tear fluid from the tear meniscus without touching the eye. This decrease in reported tear glucose concentration is likely due to our less irritating method of tear collection. The onion-stimulated tears (from a different subject) showed a seven to eight fold higher glucose concentration than in the absence of stimulation.

2.4 CONCLUSION

In the present study we demonstrate a robust ESI-MS method for determining tear glucose concentration in tear fluid. Liquid chromatography separates glucose from the majority of salts and proteins, such as lysozyme, globulin, and albumin, in tears reducing interference of the biological matrix on the sodiated-glucose signal. The resulting increase in the sensitivity and signal-to-noise ratio of glucose over data obtained without the column allows measurements of tear glucose concentrations as low as 10 μM (limit of quantification) by relying on an internal standard. We use a 1μL tear sampling method that is minimally irritating to the eye. This method enables reliable studies of physiological tear glucose concentrations in non-stimulated tears. A preliminary study indicates that the physiological, baseline tear glucose concentration is substantially below previous reports. Further studies are warranted to determine the inter- and intra-individual variation in baseline tear glucose concentration. This method will be used to establish normal values for tear glucose concentration and can monitor changes in tear glucose with changes in blood glucose. Application of this LC-MS method can help us to understand tear glucose physiology. Our future work will determine whether monitoring tear glucose concentration is a viable approach for non-invasive blood glucose monitoring. We are now using this method for tear glucose concentration measurements to examine a population of non-diabetic and diabetic subjects to determine their tear fluid glucose concentrations and their correlations of tear glucose with blood glucose.

2.4.1 Acknowledgements

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2.5 REFERENCES

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3.0 MASS SPECTRAL DETERMINATION OF FASTING TEAR GLUCOSE CONCENTRATIONS IN NONDIABETIC VOLUNTEERS

Baca JT, Taormina CR, Feingold E, Finegold DN, Grabowski JJ, Asher SA. Clin Chem 2007; 53:1370-1372.

3.1 INTRODUCTION

Glucose has been a recognized component of tear fluid since the early 1900s, but disagreement continues regarding its concentration in tear fluid and its correlation with blood glucose concentration.[1-6] Literature reports of normal tear glucose concentrations range between 0 and 9.1 mmol/L (164 mg/dL), with median values of 110 to 280 μmol/L (1.98 and 5.04 mg/dL).[1-7] In a recent study of 121 persons tear glucose concentrations ranged from below the limit of detection to 9.1 mmol/L (164 mg/dL). Much of the difference in reported tear glucose concentrations is likely due to use of different tear collection techniques.[8] Collection techniques causing severe eye irritation [such as filter paper collection[6]] are associated with the highest tear glucose concentrations, whereas less irritating techniques (such as glass capillary collection) are associated with the lowest.[2,3] Chemically stimulated tears have increased tear glucose.[8,9] Reliable tear sampling may also be confounded by individual differences in tolerance to real or expected eye stimulation during sampling.

Improved tear fluid collection, and the ability to analyze very low volumes of tear fluid, may dramatically improve measurement of tear fluid glucose concentrations and help resolve the reported discrepancies in basal tear glucose concentrations. Improved methods would also enable the study of physiologic glucose transport in the eye and advance the use of tear fluid as a surrogate for blood in measuring other clinically important analytes.

Some groups have tried to use tear glucose to diagnose diabetes,[4,5] and others have proposed continuous monitoring of blood glucose concentrations by use of contact lenses with glucose sensors.[10-13] We recently reported a photonic crystal glucose-sensing material for noninvasive monitoring of glucose in tear fluid.[10] Detailed understanding of tear glucose concentration and its regulation is critical to developing noninvasive glucose sensors.

We recently developed an ESI-MS method for measuring glucose in 1 μL samples of collected tear fluid.[9] Using this method, we studied here basal tear glucose concentrations in healthy persons without diabetes.

3.2 EXPERIMENTAL

We recruited volunteers (age 18-60 years) within and around the University of Pittsburgh. Persons with a history of diabetes were excluded. Blood and tear samples were obtained after participants had fasted overnight for at least 8 hours. The samples were always collected in the same order: capillary blood from the finger pad, tear fluid from the left eye, and then tear fluid from the right eye. This sequence was repeated 3 times for each study participant, with at least 10 minutes between successive blood sample collections. Glucose in capillary blood was

measured with an Accu-Chek® Compact glucometer (Roche), according to the manufacturer's protocol.

A total of 26 volunteers completed the study; 11 wore their usual contact lenses at the time of the study, and 15 did not wear contact lenses. The type of contact lenses worn were daily disposable (n=1), daily wear (n=6), extended wear (n=1), and silicone hydrogel lenses (n=2). Of the 15 non-contact lens wearers, 1 had a history of contact lens use, but did not wear contacts on the day of the study. For 1 contact lens wearer, the collected tear samples were lost because of vial breakage before analysis. For 2 participants (1 from the contact lens group and 1 from the non-contact lens group), 1 of the 6 tear glucose samples was lost because of instrument failure. The tear glucose concentration statistics for these 2 participants were determined from only 5 samples. All clinical procedures were approved by the University of Pittsburgh School of Medicine IRB, and all participants signed a detailed informed consent form.

Tear fluid samples of 1 μL were collected and analyzed by LC electrospray ionization mass spectrometry (ESI-MS) as previously reported.[9] Three aliquots of each tear sample were injected into the analyzer to determine the mean glucose concentration. Our study found much lower tear fluid glucose concentrations for healthy individuals than did previous studies (Figure 3.1).

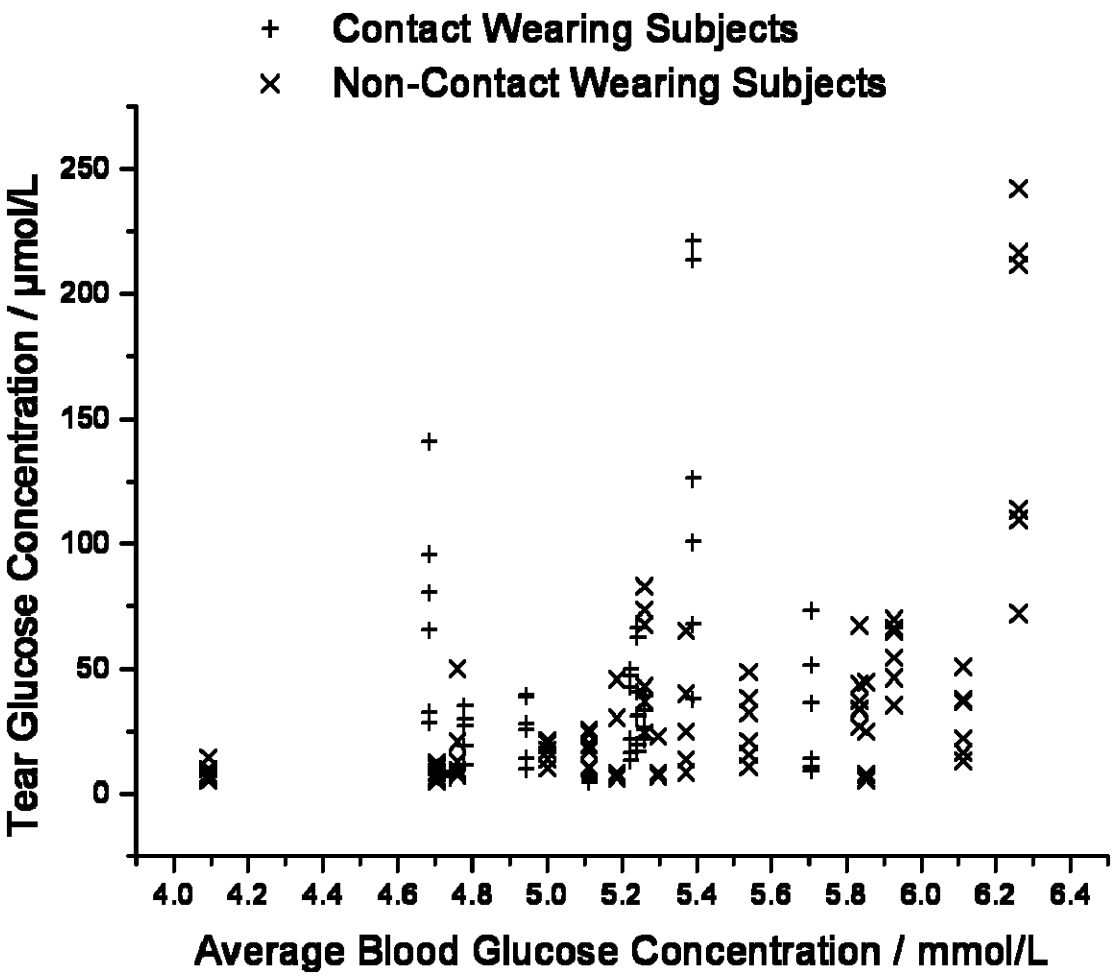

Figure 3.1. The six tear glucose concentrations determined for each subject are plotted against the subject's average blood glucose concentration. All tear glucose measurements for a given subject are hence in vertical alignment. Large variations occur in the value of the tear glucose concentrations for each subject between their eyes and between subsequent measurements of each eye over the course of the study (30-40 minutes). It should be noted that in all cases blood glucose concentration remains essentially constant over the measurement interval as subjects had fasted overnight. The average relative standard deviation of the three blood glucose measurements for each subject was 4%.

3.3 RESULTS AND DISCUSSION

The population median (range) of the tear glucose concentrations were 28 (7–161) μmol/L [0.50 (0.13–2.90) mg/dL]. The distribution of mean tear glucose concentrations was highly skewed; <28 μmol/L (0.50 mg/dL) in 50% of the study participants and <42 μmol/L (0.76 mg/dL) in 80%. Two individuals were observed rubbing their eyes during the course of the study, and they had the highest mean (SD) tear glucose concentrations: 128 (75) and 161 (71) μmol/L or 2.31 (1.35) and 2.90 (1.28) mg/dL.

Because the SD of the mean tear glucose concentration for each participant was proportional to the mean for each participant, a natural log transformation was applied to the tear glucose concentration values (Figures 3.2 and 3.3). After transformation, standard statistical methods were applied.

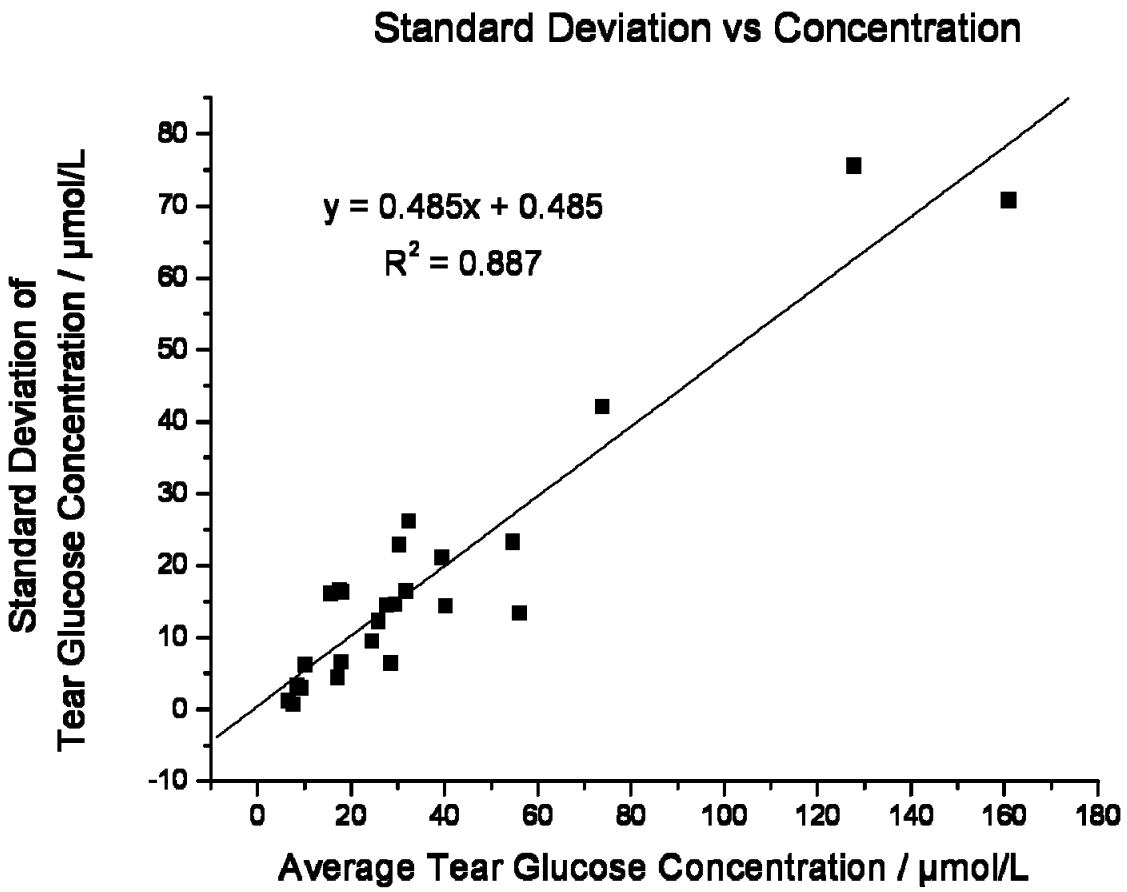

Figure 3.2. The standard deviations of the six tear fluid glucose measurements for each subject are linearly proportional to the mean value measured (~50% of the mean value). After natural log transformation, standard statistical analysis methods can be applied to the transformed data.

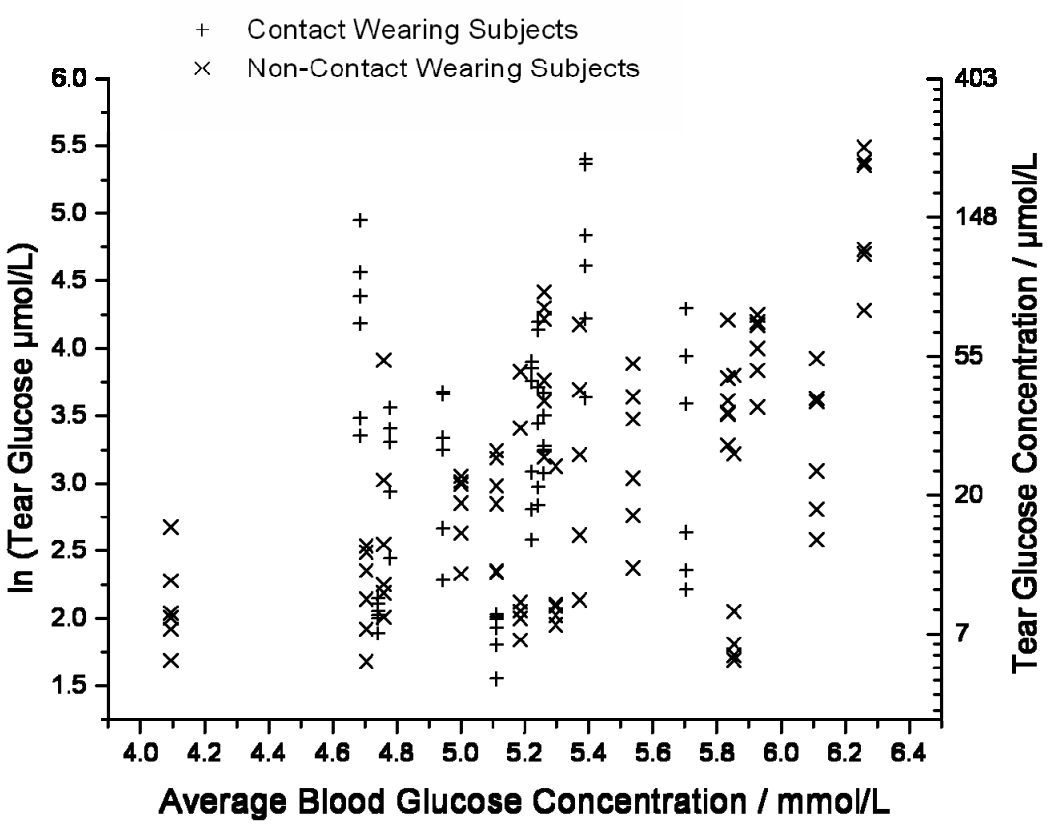

Figure 3.3. The transformed tear glucose values of the six tear glucose determinations for all subjects are plotted against the subject's average blood glucose concentration. There is significant variation in the tear glucose concentration measured in individual subjects over the course of the study (30-40 minutes). Note that all tear glucose measurements for a given subject are in vertical alignment. The average natural log transformed tear glucose value for non-contact-wearing subjects was 3.1 ± 0.8, and 3.2 ± 0.8 for contact-wearing subjects (mean ± SD).

Contact lens use did not affect mean tear glucose (p=0.715). Transformed tear glucose concentrations were significantly correlated with mean blood glucose (R=0.50, p=0.01, Figure 3.4a). This correlation for fasting tear and blood glucose is similar to that reported by Daum and Hill[1] of R=0.53 for blood and tear glucose measurements variations throughout the day. However, they reported a mean (SD) population tear fluid glucose concentration of 420 (355) µmol/L or 7.57 (6.40) mg/dL.

The glucose concentrations in the right and left eyes were highly correlated within individuals (Figure 3.4b). There was no evidence that eye-to-eye variation in individual study participants differed significantly from variation in a single eye over time. Furthermore, we observed no evidence that variation in tear glucose concentration between individuals with similar blood glucose concentrations was greater than variation in tear glucose concentration within an individual.

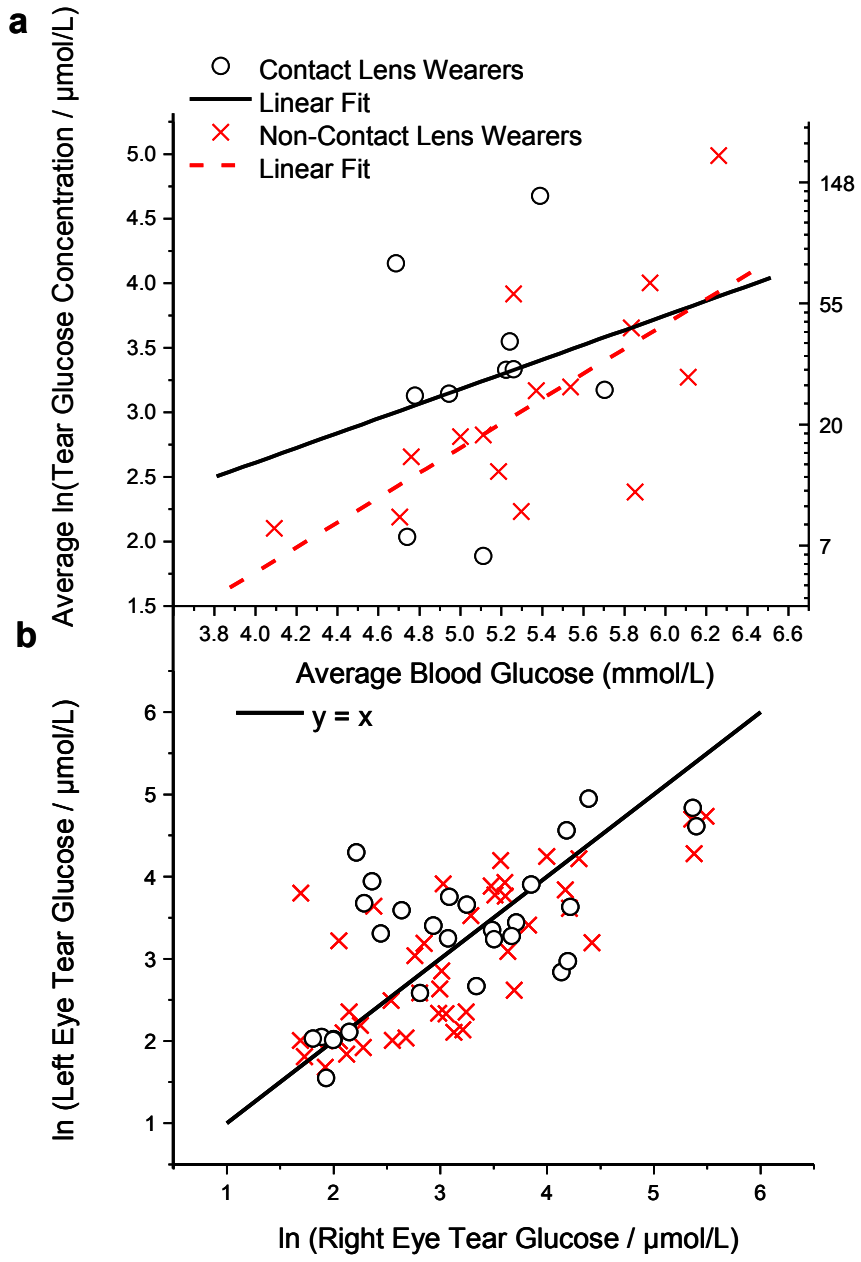

Figure 3.4. a) Correlation (p = 0.01) between the mean ln(tear glucose concentration) and the mean blood glucose concentration A linear regression for all subjects gives y = 0.80x – 1.07 (R = 0.50). Linear regression for the subpopulations of contact wearers and non-contact wearers gives y = 0.570x + 0.332 (R = 0.22) for contact lens wearers and y = 0.961x - 2.079 (R = 0.70) for non-contact wearers. b) Paired tear glucose observations within study participants. No significant differences were observed between right and left eyes (p = 0.76 2-tailed paired t test), but results may differ at any given time. The left and right tear glucose determinations that are closest in time are plotted against each other. There are hence 3 data points for each study participant.

The correlation between the transformed tear glucose value and the average blood glucose concentration appears to be stronger for non contact lens wearers than for subjects wearing contact lenses (R=0.70 vs. R=0.22). However, analysis of covariance does not indicate a significant difference (p=0.63).

The ESI-MS method used here enables reliable determination of basal tear glucose concentrations in fasting individuals. Study participants had fasted overnight, and had stable blood glucose concentrations over the brief course of the study. We observed much lower basal tear glucose concentrations than previously reported for healthy individuals. This difference is likely due to our sampling methods, which were less irritating than earlier methods that stimulated tear production chemically or with filter paper.[8] If our collection method had caused significant irritation, we would have expected tear glucose concentrations to increase over the course of the study. We observed no evidence of such an increase (Figure 3.5).

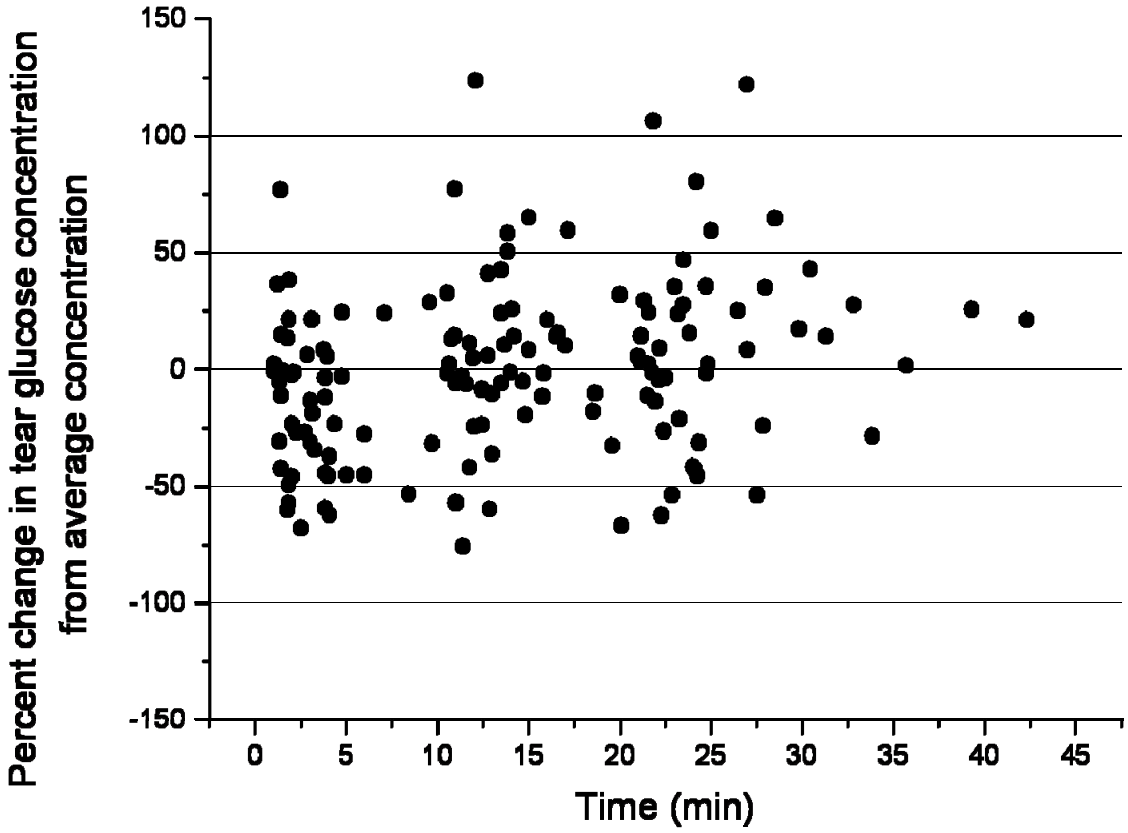

Figure 3.5. A plot of the relative difference in tear glucose concentration from the average tear glucose value for each subject versus time does not show a significant trend. If the sampling procedure caused significant irritation to the eye, we would expect an overall relative increase in tear glucose concentration with time.

We observed significant variation in tear glucose concentrations between the 2 eyes of individual study participants, as well as over time within a single eye. During this study we were cognizant of potential confounding events such as yawning and eye rubbing. Although the 2 individuals who rubbed their eyes during the study had the highest glucose concentrations, events like these do not explain the large within-individual differences observed.

Variations in tear glucose concentrations within a single individual must derive from the sum of the biological variance and any variances associated with sampling and measurement.

The error in our tear fluid collection volumes was negligible[9]; the SD observed for an individual was ~3 times the SD of the 3 replicate mass spectral measurements of a single tear fluid sample. The relative SD of these three replicate varies somewhat with glucose concentration but has a median value of 14%. Thus, the observed SD in tear glucose measurements derives mainly from actual variations of the glucose concentration in the different tear fluid samples.

We did not observe a systematic increase over time in glucose concentration variations that could result from the effect of tear depletion during repeated measurements. Tear glucose concentration appeared to vary randomly over the repeated sampling events.

3.4 CONCLUSION

We observed variations in tear glucose concentrations among fasting individuals and a significant correlation between *ln*(tear glucose concentration) and blood glucose concentration. Mean fasting tear glucose concentrations did not differ significantly in relation to contact lens use. Further studies are needed to investigate the apparent difference in the correlation between tear glucose and blood in these subpopulations.

The extremely low glucose concentrations in tear fluid, more than 100 times lower than in blood, raise questions about the physiologic role of tear glucose. Future studies are needed to address the correlation between tear and blood glucose in hypoglycemic and hyperglycemic states and in the presence of diabetes.

3.4.1 Grant/funding support

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3.4.2 Financial Disclosures

Sanford A. Asher is the scientific cofounder of Glucose Sensing Technologies LLC, a company developing glucose-sensing contact lenses.

3.4.3 Acknowledgements

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Baca JT, Finegold DN, Asher SA. Clinica Chimica Acta 2007; Submitted

4.0 TEAR GLUCOSE ANALYSIS FOR THE NON-INVASIVE DETECTION AND MONITORING OF DIABETES

Baca JT, Finegold DN, Asher SA. The Ocular Surface 2007; Submitted

4.1 INTRODUCTION

According to the most recent statistics available, approximately 20.8 million people in the United States, or about 7% of the population currently has diabetes.[1] Worldwide, over 180 million people have diabetes, and the prevalence of this disease is expected to double by 2030.[2] The economic cost of diabetes in the United States alone is about $132 billion or about 10% of healthcare expenditures.[3]

The Diabetes Control and Complications Trial clearly demonstrated that tight glycemic control is critical in managing diabetes and preventing complications such as retinopathy, nephropathy, and neuropathy.[4] In order to control blood glucose levels, current standards of care require self-monitoring of glucose several times a day, with increased frequency for patients receiving insulin.[5] Currently, self-monitoring of blood glucose requires a finger-stick blood sample for direct measurement of glucose. While many non-invasive approaches to blood glucose monitoring have been investigated, none have been able to replace the direct measurement of glucose.[6] Implantable, continuous glucose sensors have shown promise for

improving glycemic control.[7,8] However, currently approved devices must be calibrated at least twice a day with a direct blood measurement, and must be replaced after 3-7 days. Non-invasive glucose monitoring approaches under development include IR spectroscopy,[9,10] Raman spectroscopy,[11,12] measurement of optical polarization rotation,[13] photoacoustic phenomena,[14] optical coherence tomography,[15] fluorescence measurements,[16,17] surface-plasmon resonance in nanoparticles,[18] and electrical impedance measurements.[19] A single non-invasive device for monitoring blood glucose, the GlucoWatch (Cygnus, Redwood City, CA), has been approved by the FDA for supplementary blood glucose monitoring. However, results from the GlucoWatch must be frequently checked against direct blood glucose measurements. Additionally, recent studies have found that adding GlucoWatch monitoring to standard self-monitoring of blood glucose did not result in improved glycemic control.[20]

One approach to non-invasive monitoring of blood glucose is to monitor the concentration of glucose in a more accessible surrogate fluid such as tears. Tear glucose in diabetic patients has been studied for over 80 years.[21] The fairly recent development and widespread use of contact lenses has motivated ideas for using contact lenses in tear glucose analysis for the detection or monitoring of diabetes. At least three independent groups have developed glucose sensors that can be incorporated into contact lenses.[22-25] The safety of daily wear soft contact lenses in diabetic patients has recently been demonstrated,[26,27] suggesting that contact lens monitoring is potentially a viable supplement or alternative to blood glucose monitoring. In order for this approach to be successful a detailed understanding of how blood and tear glucose concentrations correlate and the underlying physiology of glucose secretion into tears are necessary. Unfortunately, there is at present, significant disagreement as to the absolute

concentrations of tear glucose in normal and diabetic subjects, as well as to whether tear glucose concentrations correlate with blood glucose concentrations.

Reported values for tear glucose in normal individuals range from 0 to 3.6 mM (65 mg/dL),[28] while concentrations as high as 84 mg/dL (4.7 mM) have been reported for diabetic persons.[21] The reported tear glucose concentrations are generally lower when the analytical techniques require smaller tear volumes. Recently, median glucose concentrations of 89 μM have been measured in 5 μL tear samples,[29] while concentrations of 28 μM have been measured in 1 μL tear samples,[30] all from fasting, non-diabetic individuals. While older studies generally measured glucose in larger volumes of chemically or mechanically stimulated tears, most recent studies specifically try to avoid conjunctival irritation and tear stimulation (Figure 4.1).

While the effect of the collection method on the tear glucose concentration has been considered,[31] there has been little clarification of the discrepancies in reported tear glucose concentrations. The recent interest in developing in situ tear glucose monitors has motivated this review of the current state of glucose sensing in tear fluid.

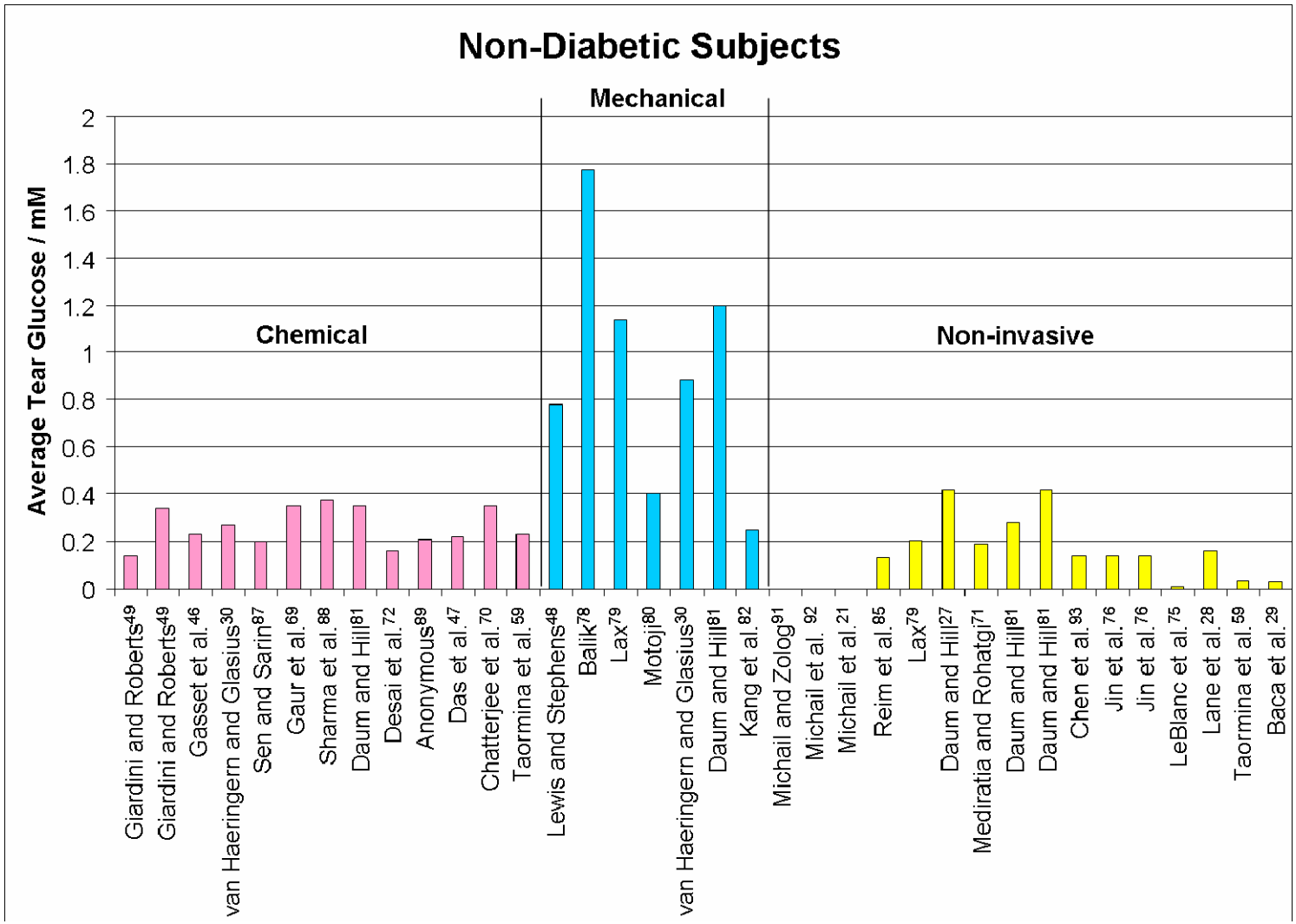

Figure 4.1. Summary of average tear glucose concentrations found in non-diabetic subjects from 1930 to present. Studies are grouped by the type of stimulation used to induce tearing (chemical, mechanical, and non-invasive). These are arranged in chronological order from left to right within each section. The details of each study are discussed in the tables and text below. Note that studies employing mechanical stimulation measure the highest tear glucose concentrations.

4.2 TEAR PRODUCTION AND GLUCOSE TRANSPORT

The tear glucose concentration is determined by the amount of glucose and the volume of the aqueous tear fraction. Changes in the transport of water in and out of the tear film have as much or greater effect on the tear glucose concentration as changes in glucose transport into the tear film. The structure and function of the tear film, especially as these relate to the regulation of the aqueous tear volume is central to the determination of tear glucose concentration.

The tear film on the surface of the eye is composed of several layers. Most superficially is a lipid layer that is less than 100 nm thick and serves several functions including preventing evaporation of the underlying aqueous layer and providing a smooth optical surface over the cornea.[32] This layer is comprised of sterol esters, wax esters, and many other minor lipid components.[33] These are secreted from the meibomian glands located on the margins of the eyelids, just posterior to the eyelashes. Dysfunction of these glands can lead to increased evaporation of tears from the eye, causing an increased tear osmolarity and clinical dry eye.[34] The lipid layer is compromised when contact lenses are worn; this layer may be completely absent over rigid contact lenses.[33]

Just below the lipid layer is the aqueous fraction. Measurements of the thickness of this layer over the cornea vary from 2.7 μm to 46 μm, with the most recently reported value of 3.3 μm.[35] One difficulty in measuring the thickness of this layer is that it changes immediately after a blink, thinning as it distributes over the surface of the eye.[35] In the presence of contact lenses, the aqueous tear film can be measured both in front of the contact lens (pre lens), and between the lens and the cornea (post lens). Both the pre lens and post lens tear films are measured to be

approximately 3 μm thick.[35] In both the presence and absence of contact lenses, the tear film is considerably thicker near the margins of the eyelids, where a meniscus forms. The total volume of the aqueous tear film is about 7 μL,[36] and its production and elimination are discussed below.

Below the aqueous layer of the tear film is a mucin layer, consisting of glycoproteins which lubricate the surfaces of the eye. At least 20 different mucins are present in this layer. They provide a hydrophilic surface, over which the aqueous fraction can rapidly flow.[37,38] This layer is approximately 30 μm thick[32] and its components are produced by both the cornea and conjunctiva.[39] The mucin layer moves freely over the glycocalyx, which is comprised of membrane-associated mucins bound to the cornea and conjunctiva.

4.2.1 Production and elimination of aqueous tear fluid

The rate of tear production can vary by a factor of over 100 between basal tear production and active tearing.[40] By studying the clearance of fluorescein, Mishima *et al.* estimated the average rate of tear fluid production to range between 0.5 and 2.2 μL/min (with an average of 1.2 μL/min) at baseline.[36] After the introduction of fluorescein, a rapid decrease in fluorescence was observed that was ascribed to reflex tearing. After about five minutes, the fluorescein clearance rate decreased to about 17% per minute. Calculation of the "unstimulated" tear production was based on the lower fluorescein clearance rates seen at later times.[36]

While estimates of the rate of baseline tear fluid production have not varied significantly since the 1966 report of Mishima *et al*., the relative contributions of different sources to the aqueous tear fraction continues to be debated. Traditionally, aqueous tear fluid was thought to be almost entirely produced by the main lacrimal gland with very minor contributions from the accessory lacrimal glands and goblet cells in the conjunctiva.[41] Recent studies, however, have

shown that the rate of water flow across the conjunctiva can be as high as 1-2 μL/min and may account for a large proportion of basal tear production.[42] While estimates of the contribution of conjunctival secretion to basal tear fluid production vary widely, recent models of tear production suggest that 25% of tear fluid is produced by the conjunctiva in the absence of reflex tearing.[43] While the aqueous fraction in stimulated tears is derived primarily from the lacrimal glands, the aqueous fraction in unstimulated tears may have a significant conjunctival source.

The elimination of the aqueous fraction of tears can also influence the concentration of tear fluid analytes. The major routes of elimination for aqueous tears are drainage through the lacrimal canaliculi to the nasolacrimal duct and evaporation.[41] Under certain conditions, conjunctival or corneal absorption of water can contribute to the elimination of aqueous tears.[43] During a blink, tears are swept from the lateral canthus toward the medial canthus.[44] Tears then pass into the lacrimal canaliculi, which connect to the lacrimal sac. The tears then drain by gravity through the nasolacrimal duct and into the inferior nasal meatus.[44] Blockage of this drainage system by cautery or with plugs increases the basal tear volume, and is a common treatment for dry eyes.[34]

The rate of tear film evaporation is proportional to the surface area of the eye exposed to the environment.[45] The palpebral fissure, *i.e.* the distance between the upper and lower eyelids, varies between subjects. It can also be increased by lid surgery or by disease states, such as hyperthyroidism.[34]

Disturbances of the lipid tear film can lead to dramatically increased rates of evaporation. When the lipid layer is washed from the eye or the meibomian glands are occluded, the rate of evaporation increases from 0.1 μL/min to 1.7 μL/min.[32] Rates of evaporation also depend on the ambient temperature and humidity.[32] As noted above, the lipid layer can be compromised by

contact lenses; increased tear evaporation is observed during contact lens use.[40] This increase in evaporation does not appear to vary significantly with contact lens composition or level of hydration.[46] The average increase in the rate of evaporation during contact lens wear is about 35%, however the rate of evaporation may vary significantly between subjects.[46]

4.2.2 Glucose transport in tear fluid

The source of glucose in tears remains unclear. Explanations for the mechanisms of glucose transport into the tear fluid have changed as different tear glucose concentrations were observed. The studies that used mechanically irritative methods to obtain tear fluid found the highest glucose concentrations. These studies also found a correlation between blood and tear glucose concentrations.[31,47-49] Mechanical irritation abrades the conjunctiva and probably results in the leakage of glucose from epithelial cells or the interstitial space directly into the tear fluid.[31,41]

Studies that attempt to avoid abrasion of the cornea and stimulation of tearing measure the lowest glucose concentrations.[29-31] It has been suggested that exclusion of glucose from tear fluid aids in preventing bacterial infections of the exterior eye.[50] Many of the studies of chemically stimulated tears find very low concentrations of glucose, suggesting that very little, if any, glucose comes from the lacrimal glands.[31,48,50]

There is only limited evidence for glucose transporters in the tear glands, the conjunctiva, and the cornea. The constitutive glucose transporter, GLUT-1, is present in the apical corneal epithelium,[51] but absent in the lacrimal glands and conjunctiva.[52] Expression of GLUT-1 in the corneal epithelium is increased after corneal abrasion, and appears to have a role in corneal wound healing.[53] A sodium/glucose cotransporter, SGLT-1, is present on the apical side of the

bulbar and palpebral conjunctiva.[54,55] This transporter operates in both directions, allowing both secretion and absorption of glucose, depending on sodium and glucose concentrations.[56] While this transporter removes glucose from the tear fluid under physiological conditions,[57] it can add glucose to the tear fluid during the hypoosmotic stress that occurs when rinsing the eye with water or after swimming.[28,43,56,57]

Recent models of electrolyte and metabolite transport in the tear fluid suggest that a paracellular transport mechanism is required to fully explain observed electrolyte concentrations.[43] Studies of polyethylene glycol oligomer permeability in the cornea and conjunctiva of rabbits suggest that there are paracellular pores with diameters of ~ 4 nm and ~ 2 nm in the conjunctiva and cornea respectively.[58] While glucose should be able to pass though these pores and into the tear fluid, its transit has not been directly measured. While the distribution and regulation of glucose transporters affecting the tear glucose concentration is not yet fully characterized, glucose transport across the conjunctiva appears to be the major determinant of tear glucose concentration in the absence of reflex tearing.

4.2.3 Stimulated tears

Increased aqueous tear volumes can be caused by increased tear production (lacrimation) or by decreased tear elimination, which may be caused by obstruction of the tear drainage system or paralysis of the muscles responsible for sweeping tears towards the puncta.[44] Some authors have questioned the existence of a true basal tear production rate, suggesting that all tears are "stimulated."[59] This argument is based on the fact that the rate of tear production decreases dramatically with the administration of anesthesia. However, while tear production from the lacrimal gland may require stimulation, the epithelial cells of the conjunctiva continuously

secrete water and ions in response to electrolyte and metabolite concentration gradients.[57] The rate of aqueous tear production can increase over 100-fold in response to a variety of physical or chemical stimuli; the changes in aqueous tear production may alter the chemical composition of tear fluid.[40]

In a 1981 review of tear biochemistry, Van Haeringen recounted several methods of inducing tearing in order to increase the sample volume.[41] Chemical stimulants of lacrimation include onion vapors, ammonia, formalin, benzyl bromide, chloracetophenone, and bromoacetone.[41] Cigarette smoke and onion vapors have also been used to stimulate tearing.[41,60] Mechanical stimulation of the conjunctiva or cornea with absorbent materials such as filter paper, cellulose sponges, and cotton thread is also commonly used to increase tear production.[41,47,49] Because these methods can abrade the conjunctiva, which could alter analyte concentrations, other methods of stimulating lacrimation that do not directly contact the conjunctiva have been investigated. These include stimulating the nasal mucosa, inducing a gag reflex with a tongue depressor, having subjects look into the sun, and inducing emotional tearing.[40,41,59]

Lacrimation in response to physical and chemical stimulation of the ocular surfaces or photo stimulation of the optic nerve is mediated by a reflex arc. Activation of afferent sensory nerves in the conjunctiva, cornea, nasal mucosa, or optic nerve leads to reflexive stimulation of efferent sympathetic and parasympathetic nerves innervating the lacrimal gland.[61,62] While neurotransmitters from the autonomic efferent nerves such as acetylcholine and norepinephrine are the most potent stimulants of tearing, many other minor neuromediators have been identified and recently reviewed.[62,63] Recent immunohistochemical studies suggest that there is direct parasympathetic enervation of goblet cells in the conjunctiva and direct sympathetic enervation

of stratified squamous cells in the conjunctiva.[39] Therefore, transport of fluid and mucins directly across the conjunctiva may also lead to increased tear volume and mucin secretion after mechanical stimulation of the conjunctiva.

It is clear that reflex tearing must be avoided if basal tear glucose concentration is to be accurately measured. In practice, this is very difficult because only small volumes of basal tear fluid are available. Individuals may respond differently to even the most benign collection techniques; the mere discussion of tear fluid collection is sufficient to stimulate tearing in some subjects. There may also be uncontrolled and unrecognized variables affecting tear stimulation in different subjects such as changes in room temperature or humidity from day to day. In addition to all of these issues, contact lens use can cause irritation and stimulate tear production.[40] This could alter the relevant basal tear glucose concentration measured by a contact lens-based glucose sensor. As contact wearers become habituated to their lenses, however, this irritation, as measured by blink frequency, decreases.[40]

4.2.4 Effects of diabetes on tear glucose transport

Most studies of tear physiology have been conducted on non-diabetic animals and humans. If tear glucose analysis is to be used to detect or monitor diabetes, we must consider the effects of diabetes on aqueous tear production and glucose transport in tear fluid. While the relative importance of different molecular mechanisms in diabetes pathogenesis is debated, the increased intravascular concentration of glucose that results from diabetes ultimately leads to microvascular and nerve damage.[64,65] In light of the above discussion of aqueous tear film production, damage to either the vasculature supplying blood to the eye or the nerves of the lacrimal reflex arc might be expected to alter tear production.

Keratoconjunctivitis sicca, or dry eye, can be caused by decreased tear production, or alterations in the tear fluid such as loss of the lipid layer, leading to increased tear osmolarity. Dry eye is more common in diabetic patients, and correlates with poor glycemic control as measured by mean annual hemoglobin A1c (HbA1c) levels.[66] When measured by fluorescent dye clearance, basal tear secretion rates are indistinguishable in control and diabetic subjects.[67,68] Reflex tearing, as measured by a Schirmer test without anesthesia, is decreased significantly in diabetic subjects.[67] This decrease in reflex tearing may be due to a decreased sensitivity of the conjunctiva resulting from neuropathy.[69] During episodes of hyperglycemia, increased osmolarity in the extracellular fluid may also impede aqueous tear flux across the conjunctiva or into the lacrimal gland.[66] Other differences noted in the eyes of diabetic subjects include a decreased tear break-up time, loss of mucin secreting goblet cells, and squamous metaplasia of the conjunctiva, indicating chronic irritation.[69] Overall, diabetes can effect tear composition and the structure and function of the tissues that contribute to aqueous tear production.

Increased tear glucose concentration in diabetic subjects has been repeatedly demonstrated.[21,29,47-49,70-73] However, many of these studies used filter paper to collect the tear sample, and the observed high glucose levels may be due to intercellular fluid leaking through the abraded conjunctiva.[31] A recent study of tear glucose concentration in 50 non-diabetic subjects and 33 diabetic subjects specifically tried to avoid chemical or mechanical stimulation by collecting tear samples with a glass microcapillary.[29] This study observed overlapping ranges of tear glucose concentration in fasting normal and diabetic subjects, but found a statistically significant increase in average tear fluid concentrations in the diabetic subjects (89 μM for normal and 150 μM for diabetic subjects). While tear glucose concentrations are clearly increased in diabetic subjects, the precise mechanism remains unclear. Studies using sampling

methods that cause mechanical stimulation of the conjunctiva are likely to be simply measuring analytes in direct equilibrium with intercellular fluid. Studies of non-stimulated tears may measure increased glucose due to paracellular glucose transport in the conjunctiva. It is also possible that these studies were not entirely successful in avoiding contact with the eye during sampling with glass microcapillaries over the required five minute sampling periods.[29,31]

4.3 MEASUREMENT OF TEARS IN EXTRACTED TEAR FLUID

The chemical components of tears were first studied by Fourcroy and Vauquelin over 200 years ago.[74] The first quantitative report of glucose in tear fluid appears to be from 1930, when Ridley reported tear glucose concentrations of 3.6 mmol/L (65 mg/dL).[75] While the method of glucose analysis was not described in this study, the tear volume studied was 0.2 mL, suggesting that tearing was induced to collect such large volumes. Reported values of tear glucose concentration have steadily decreased as the analytic methods required less sample volume. At the opposite extreme, LeBlanc *et al.* recently reported an average tear glucose concentration of 7.25 ± 5.47 μmol/L in five patients in an intensive care unit.[76] Glucose was measured using high performance liquid chromatography with pulsed amperometric detection. The sample volumes studied were variable and not individually reported, but were presumably less than 1 μL.[77,78] The fact that the reported values of tear glucose concentrations have varied by 1000-fold between studies demonstrates the need for careful consideration and control of experimental parameters such as collection method, analysis method, and selection of the clinical population.

4.3.1 Mechanical stimulation

Because the tear fluid collection method seems to have the greatest influence on the reported tear glucose concentrations, we have grouped the previous studies of tear glucose concentration by collection methodology. As noted above, tear collection methods that mechanically stimulate, and presumably abrade the conjunctiva, measure the highest levels of glucose (Table 4.1).

Table 4.1. Mechanical Stimulation in the Study of Tear Glucose Concentration

Year	Reference	Sample Collection method	Glucose Measurement Method	Volume of Sample [mL]	NORMAL				DIABETIC			
					Range [mM]	Average ± std [mM]	patients	n	Range [mM]	Ave± std [mM]	patients	n
1958	Lewis and Stephens[49]	Clinistix	King method	0.1	0-2.22	0.78 ± 0.50	13	18	0.56-8.30	2.90 ± 1.90	15	29
1965	Balik[79]	filter paper strips	reduction	0.1	0.25-3.3							
1969	Lax[80]	filter paper strips	enzymatic	NA		1.141 ± 0.159	32	32				
1971	Motoji[81]	filter paper strips	glucose oxidase	NA		0.40 ± 0.09	32	32		0.80 ± 0.24	169	169
1977	van Haeringern and Glasius[31]	filter paper strips	glucose dehydrogenase	NA	0.1-1.5*	0.88	10	10	2-10*	5.36	5	5
1984	Daum and Hill[82]	capillary[a]	glucose dehydrogenase	0.005	1.1-1.4*	1.2*	3	6				
1988	Kang *et al.*[83]	Schirmer strips	glucose oxidase	0.01	0.15-0.45	0.25 ± 0.10	30	30				
1993	Haeckel *et al.*[84]	Schirmer strips	NA	NA		0.056						

* Estimated from graph

[a] Mechanical rubbing of bulbar conjunctiva

These studies generally use a filter paper or Schirmer test strip to collect the tear sample. Glucose is then extracted from the filter paper and measured enzymatically,[31,83] or transferred to a glucose oxidase reagent strip and measured "semiquantitatively" by comparing the color of the strip to a calibrated color chart.[47] More recent investigations have instilled reagent strips in the lower conjunctival sac to more directly measure glucose concentration.[48,85] A limitation of these studies is that color comparison can be subjective, and the subjects could only be stratified into 4 or 5 arbitrarily defined groups.[48,49,71,72]

Van Haeringen and Glasius compared glucose concentrations in the chemically and mechanically stimulated tears of normal and diabetic subjects.[31] They first stimulated tearing with 2-chloracetophenone and collected 20 μl tear samples with a capillary tube. They then collected a second tear sample using filter paper strips. The tear glucose concentration determined using filter paper collection was higher for all subjects. The increase was between 0.1 and 1.5 mmol/L for subjects with blood glucose below 10 mmol/L (180 mg/dL) and as high as ~9 mmol/L for extremely hyperglycemic subjects with blood glucose concentrations of ~20 mmol/L (360 mg/dL). A 1969 dissertation by Lax, which is cited by Van Haeringen and Glasius, found glucose concentrations of 0.206 ± 0.027 mmol/L (mean ± SD) using capillary collection and 1.141 ± 0.159 mmol/L using filter paper collection in non-diabetic subjects.[31,80]

Daum and Hill used capillaries to collect 5 μL tear samples at different times from non-diabetic subjects after mechanical stimulation of the conjunctiva with a cotton applicator.[82] They measured an increase in tear glucose concentration from ~0.28 mmol/L (~5 mg/dL) before stimulation to a peak value of ~2.5 mmol/L (~45 mg/dL) 10 minutes after stimulation. Tear glucose concentrations remained elevated at 30 minutes, but returned to baseline at 60 minutes. This time course suggests that caution must be exercised in planning and interpreting tear

glucose studies. Unrecognized conjunctival stimulation early in a time course study of tear glucose may affect many subsequent measurements. A similar increased tear glucose concentration after corneal or conjunctival irritation has also been observed in rabbits.[86,87]

Daum and Hill also observed an increase in tear glucose after hypoosmotic stress induced by immersion of the eye in distilled water for 60 seconds.[82] It is not immediately clear whether this should be considered physical or chemical stimulation of tearing. However, the tear glucose response seems similar to that of physically stimulated tears.

Studies of mechanically stimulated tears generally find a correlation between the tear and blood glucose. This is not surprising as the glucose measured in these studies likely comes directly from the interstitial space in the conjunctiva. Rabbit studies suggest that short term contact lens use increases tear glucose concentrations in a manner similar to other means of mechanical stimulation.[40,87] However, it should be noted that our recent study found no evidence of increased tear glucose concentrations in fasting subjects wearing contact lenses.[30]

4.3.2 Chemical and non-contact stimulation

Early analytical techniques used to study tear glucose concentration required analyte volumes that would take hours to collect at basal rates of tear secretion. Hence, many studies have used a chemical lachrymator to induce tearing and speed sample collection (Table 4.2).

Table 4.2. Chemical Stimulation in the Study of Tear Glucose Concentration

Year	Reference	Inducing agent	Sample Collection Method	Glucose Measurement Method	Volume of Sample [mL]	NORMAL†				DISEASE STATE†			
						Range [mM]	Avg ± std [mM]	Pa-tients	n	Range [mM]	Avg ± std [mM]	Pa-tients	n
1950	Giardini and Roberts[50]	tear gas	pyrex tubes	Hagedorn-Jensen	0.22	0.16-0.43	0.34 ± 0.07	12	25				
				fermentation methods	0.22	0.056-0.28	0.14 ± 0.07	12	25				
1968	Gasset *et al.*[47]	spirit of ammonia	capillary	enzymatic	0.005-0.020		0.23 ±0.17	28	50		1.53 ± 0.8‡	26	26
1977	van Haeringern and Glasius[31]	2-chloraceto-phenone	capillary	glucose dehydrogenase	0.020	0.1-1.0*	0.27	10	10	0.5-1.0*	0.76	5	5
1980	Sen and Sarin[88]	reflex	NA	glucose oxidase	0.1-0.2		0.20 ± 0.14	50	50		0.92 ± 0.52[d]	50	50
1982	Gaur *et al.*[70]	spirit of ammonia	capillary	NA	NA		0.35 ± 0.06	50	50		0.74 ± 0.16[e]	25	25
											1.20 ± 0.08	50	50
1983	Sharma *et al.*[89]	spirit of ammonia	capillary	King and Asatoor	0.05	0.18-0.67	0.38± 0.14	35	35				
							0.43± 0.04[c+]	25	25		1.05 ± 0.19[c]	25	25
							0.39± 0.07[a+]	25	25		0.90± 0.20[a]	25	25
1984	Daum and Hill[82]	allium cepa, onion	capillary	glucose dehydrogenase	0.005	0.3-0.4*	0.35*	3	10				
1987	Desai *et al.*[73]	ammonium hydroxide	steel cannula	glucose oxidase	> 0.2	0-0.28	0.16± 0.11	25	25	0-1.6	0.67± 0.05	40	40
1988	Anonymous[90]	ammonium hydroxide	NA	glucose oxidase	0.13	0.15-0.26	0.21± 0.04	15	15	0.39-1.22	0.75± 0.23	10	10
1995	Das *et al.*[48]	spirit of ammonia	capillary	glucose oxidase	NA		0.22± 0.04				1.00 ± 0.22		
2003	Chatterjee *et al.*[71]	spirit of ammonia	dextrostix	glucose oxidase	NA	0.056-1.00	0.35 ± 0.24	76	76	1.2-1.8	1.58 ± 0.17	52	52
										2.1-3.8	2.68 ± 0.57	44	44
										4.2-5.3	4.67 ± 0.39	16	16
										7.0-7.9	7.44 ± 0.67	8	8
										9.4-10.2	9.78 ± 0.44	4	4
2007	Taormina *et al.*[60]	allium cepa, onion	capillary	ESI-MS	0.001		0.211 ± 0.008	1	6				

† All disease states reflect diabetes and all normal states reflect normal health, except "a" signifies acute conjunctivitis, "a+" signifies treatment for acute conjunctivitis; "c" signifies corneal ulcer, and "c+" signifies treatment for a corneal ulcer.

‡ Data listed for highest level of glucose observed

[d] Chemical diabetes; [e] Uncontrolled diabetes

***** Estimated from graph

The concentration of many analytes is known to vary with tear secretion rate. Protein and calcium concentrations decrease as the flow rate increases, while potassium concentrations increase with the flow rate.[41] In a 1984 study, Daum and Hill collected 5 μL tear samples every 20 seconds for 2 minutes after non-diabetic subjects were exposed to raw onion (Allium Cepa) vapors for 30 seconds.[82] Tear glucose concentration decreased monotonically from 0.3 mmol/L (6 mg/dL) before tearing, to 0.1 mmol/L (2 mg/dL) at 2 minutes after exposure.

Comparison between studies that used chemically stimulated tears is difficult, as different lachrymators are used and the precise timing of tear collection is rarely reported. Some of these agents have been shown to cause corneal and conjunctival edema and epithelial erosion or ulceration.[91] Hence, long term exposure to lachrymators may cause a significant *increase* in glucose concentration as the physical barriers of the ocular surface are compromised. Use of lachrymators is also likely to cause subjects to rub their eyes, which we have anecdotally found to correlate with increased tear glucose concentrations in our recent studies.[30]

Overall, the average tear glucose concentrations measured in studies of chemically stimulated tears fall within the range of tear glucose concentrations measured in non-stimulated tears. Many studies of chemically stimulated tears did not find a general correlation between tear and blood glucose for all subjects.[50] However, the ability to broadly classify subjects as diabetic or non-diabetic through tear glucose measurement, was often demonstrated in these studies, especially when postprandial samples were considered.[48,71]

4.3.3 Non-stimulated tears

As more sensitive analytical methods have been developed, investigators have attempted to study glucose concentrations in non-stimulated or basal tears (Table 4.3).

Table 4.3. The Study of Glucose Concentration in Non-Stimulated Tears

Year	Reference	Sample Collection Method	Glucose Measurement Method	Volume of Sample [mL]	NORMAL				DIABETES			
					Range [mM]	Average ± std [mM]	Pa-tients	n	Range [mM]	Average ± std [mM]	Pa-tients	n
1937	Michail and Zolog[92]	capillary	NA	NA	0	0	8	8				
1937	Michail *et al.*[93]	capillary	Hagedorn-Jensen	NA	0	0	8	8				
1937	Michail *et al.*[21]	capillary	Hagedorn-Jensen	NA	0	0	5	5	1.78-4.89	3.311 ± 0.906	12	12
1967	Reim *et al.*[86]	capillary	enzymatic	NA		0.13						
1969	Lax[80]	capillary	enzymatic	NA		0.206 ± 0.027	43	43				
1982	Daum and Hill[28]	capillary	glucose dehydrogenase	0.005	0-3.43	0.42 ± 0.36	12	875				
1983	Mediratia and Rohatgi[72]	capillary	glucose oxidase	0.013	0.11-0.33†	0.19 ± 0.04†	30	30	0.67-1.60	1.10 ± 0.26	10	10
1984	Daum and Hill[82]	capillary	glucose dehydrogenase	0.005	0.06-0.90*	0.28[a]	8	48				
						0.42[b]	8	548				
1996	Chen *et al.*[94]	capillary	glucose oxidase	0.0001-0.0005	0.128-0.166	0.139 ± 0.014	6	6				
1997	Jin *et al.*[77]	capillary	CE-LIF	0.0002-0.0005		0.137 ± 0.013[c]; 0.139 ± 0.003[d]	1	1				
2005	LeBlanc *et al.*[76]	capillary	HPLC-pulse amperometry	NA					0.0008-0.021*	0.00725 ± 0.005‡	5[e]	44
2006	Lane *et al.*[29]	capillary	HPLC-pulse amperometry	0.005	0-5.7	0.16 ± 0.03‡	50	50	0-9.1	0.35 ± 0.04‡	33	33
2007	Taormina *et al.*[60]	capillary	ESI-MS	0.001	0.013-0.051	0.032	1	12				
2007	Baca *et al.*[30]	capillary	ESI-MS	0.001	0.007-0.161	0.028[f]	25	25				

† Average concentration and range for all age groups
‡ Mean given
* Estimated from graph
[a] Prolonged closed eyes, [b] open eyes
[c] on column, [d] precolumn
[e] Critically ill patients receiving insulin, where two had a history of diabetes
[f] Median given

Non-stimulated tear samples are generally collected with a capillary by gently touching it to the tear film meniscus. While Daum and Hill trained subjects to collect their own tears throughout the day,[28,82] most investigators have conducted these investigations by collecting tear samples in a clinical setting.

One of the first large studies of non-stimulated tears measured an average tear glucose concentration of 0.42 ± 0.356 mmol/L in 875 tear samples from 12 non-diabetic subjects.[28] This average tear glucose concentration was somewhat higher than more recent measurements of chemically stimulated tears. While studies using lachrymators could be measuring artificially low tear glucose concentrations, it is also possible that some mechanical stimulation was involved in the collection of basal tears in this study. The glucose dehydrogenase method used in this study required sample volumes of 5 μL, which would have required sample collection times of at least 5 minutes. Even if mechanical stimulation were avoided during sampling, increased evaporation due to the prolonged suppression of blinking could alter the glucose concentration. One of the interesting results of this study was the observation of diurnal variations in tear glucose that roughly track changes in blood glucose.

One of the largest and most recent studies of tear glucose concentration was reported by Lane *et al.* in 2006.[29] This study monitored tear glucose concentration in 73 non-diabetic subjects and 48 diabetic subjects before and after an oral glucose bolus. These groups were farther divided into fasting and non-fasting groups. The 5 μL tear samples collected at each time point were analyzed with a liquid chromatography-pulsed amperometric detection method. Average tear glucose concentration in non-diabetic subjects was 0.16 ± 0.03 mmol/L while average tear glucose concentration in diabetic subjects was 0.35 ± 0.04 mmol/L (mean ± standard error). Individual glucose determinations, however, varied from below the limit of

detection to over 9.1 mmol/L. Lane *et al.* were able to show a modest correlation between average tear and blood glucose concentrations at the five time points in the study. Unfortunately, only results averaged over the subject population were given. Thus there is no indication of how well tear and blood glucose concentrations correlate within individual subjects. The use of 5 μL tear samples may have precluded the study of truly non-stimulated tears for the reasons noted previously.

A few recent studies analyzed microliter or submicroliter volumes of tears.[30,60,76,77,95] The previously mentioned study of critically ill patients attempted to assess the feasibility of monitoring tear glucose instead of blood glucose in the intensive care unit.[76] The investigators obtained 44 simultaneous blood and tear samples from 5 sedated subjects who were receiving insulin, two of whom had a history of diabetes. This study measured the lowest average tear glucose concentration (7.25 ± 5.47 μmol/L) of any study where glucose was detected in tears. Despite a wide range of blood glucose concentrations, the study did not detect a clinically useful correlation between these values. These results in critically ill patients are of little use in predicting whether tear fluid analysis can be used to monitor glucose in healthy people. However, it is clear that tear glucose monitoring with this method in the ICU is not a feasible replacement for blood glucose monitoring.

A recent study in our laboratory found a median (range) tear glucose concentration of 28 (7–161) μmol/L or 0.50 (0.13–2.90) mg/dL in 25 fasting subjects.[30] The mean (standard deviation) tear glucose concentration was 37 ± 37 μmol/L. We found a highly skewed distribution of tear glucose values; tear glucose concentrations were less than 42 μmol/L in 80% of subjects. We found no statistically significant difference between contact lens wearers and non-wearers. Linear regression showed a modest correlation between tear and blood glucose

concentrations (R = 0.5). We compared tear glucose concentrations within subjects over 30 minutes and did not see any significant trend with time, suggesting that conjunctival irritation was minimized or eliminated. In our study, glucose concentrations were measured using liquid chromatography and electrospray ionization mass spectrometry. Due to the fact that we collected only 1 μL tear samples, studied non-diabetic fasting subjects, and observed no evidence of conjunctival stimulation, we believe this to be one of the most precise studies of baseline tear glucose.

As described in detail above, there is very little reliable information on whether glucose concentrations in unstimulated tears track blood glucose concentrations. While previous studies showed correlations between averaged tear and blood glucose concentrations, there is little direct information about the existence of such a correlation within individual subjects. We recently used our mass spectrometry method to investigate the relationship between blood and tear glucose concentrations in subjects during a glucose tolerance test (oral administration of 75 grams of glucose). Tear samples of 1 μL were collected and analyzed as previously reported, and great care was taken to avoid abrasion of the conjunctiva.[60] Figure 4.2 shows the blood and tear glucose concentrations over time for a non-diabetic subject.

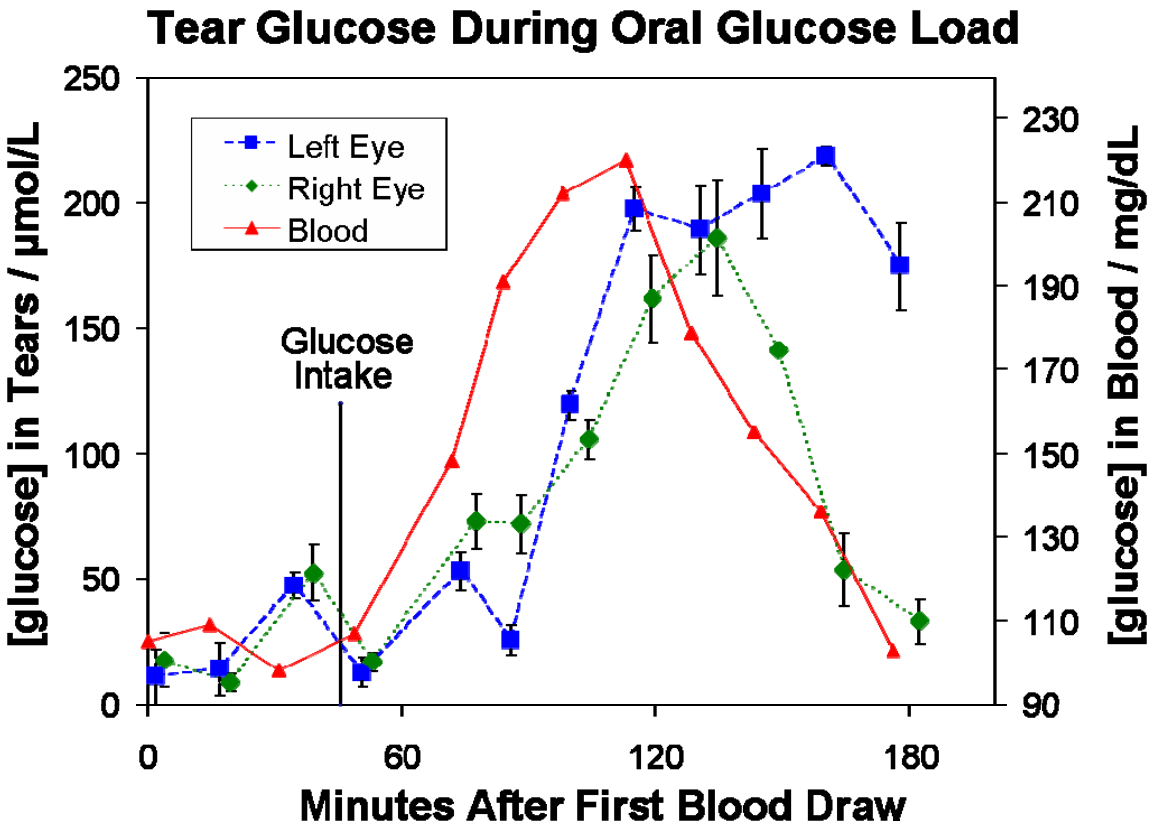

Figure 4.2. Tear and blood glucose concentrations in a non-diabetic, male subject. Blood glucose concentration doubles by ~70 minutes after glucose ingestion.

Tear glucose concentrations appear to track blood glucose concentrations in this subject with a lag time of ~20 minutes. The tear glucose concentration in the left eye remains elevated even after the blood glucose concentration decreases. While this may be the true physiological response for this subject, it could also be explained by an unrecognized irritation of the left conjunctiva during the latter half of the experiment (140-160 min). An essential point is that while the blood glucose concentration increases ~2-fold, the left and right eye tear glucose concentrations increase ~7-fold. This demonstrates a complex relationship between tear and blood glucose concentrations for this subject.

Figure 4.3 shows a similar plot of blood and tear glucose concentrations during a glucose tolerance test for a diabetic subject.

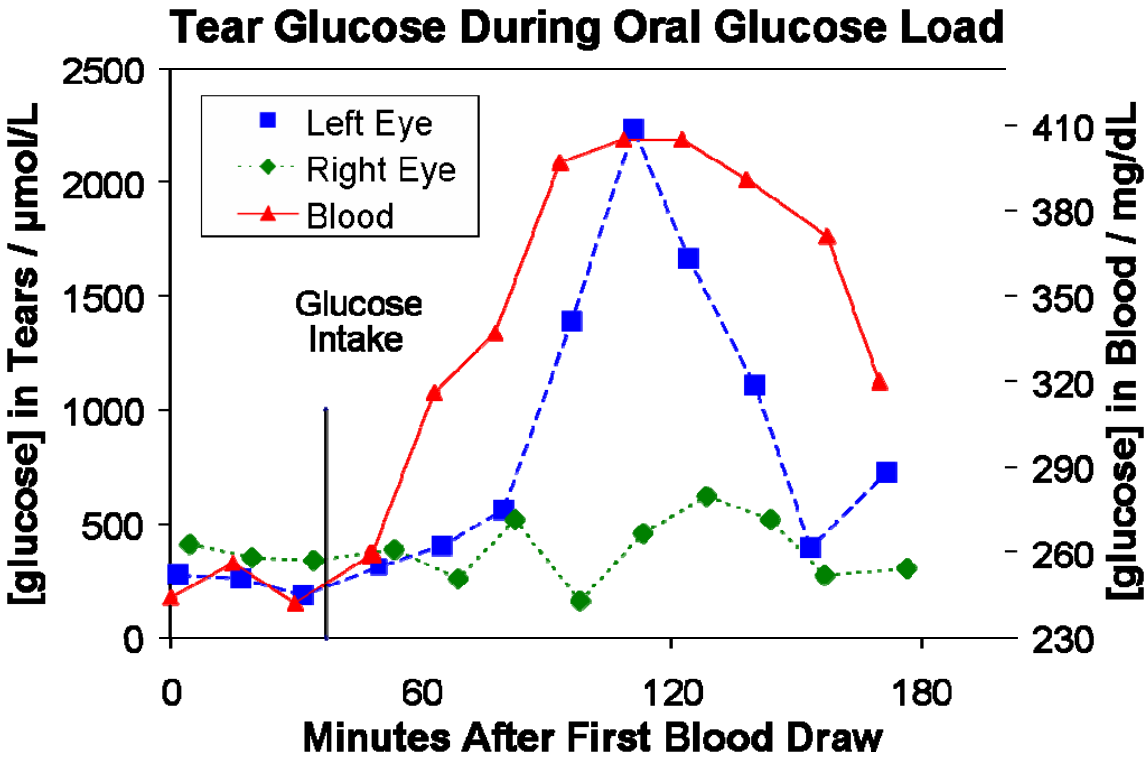

Figure 4.3. Tear and blood glucose concentrations in a diabetic, female subject. Blood glucose concentration peaks at ~80 minutes after glucose ingestion with a ~60% increase.

Blood and tear glucose concentrations are clearly higher for the diabetic subject at all times. Prior to glucose ingestion, the blood glucose concentration in this subject was about twice as high as for the non-diabetic subject of Figure 4.2. However, the basal tear glucose concentration of the diabetic subject is about ten times higher than for the non-diabetic subject.

The extraordinary ~5 fold increase in tear glucose concentration in the left eye differs completely from the change in the right eye, where (excepting a single point at ~95 minutes) the ~60% increase in tear glucose concentration is roughly proportional to the increase in blood glucose concentration. It is possible that the dramatic increase in left eye tear glucose concentration results from unrecognized conjunctival irritation, causing interstitial glucose to leak into the tear fluid.

A replicate of the glucose tolerance test shown in Figure 4.2, with the same non-diabetic subject, demonstrates the challenge of determining a correlation between tear and blood glucose concentration (Figure 4.4).

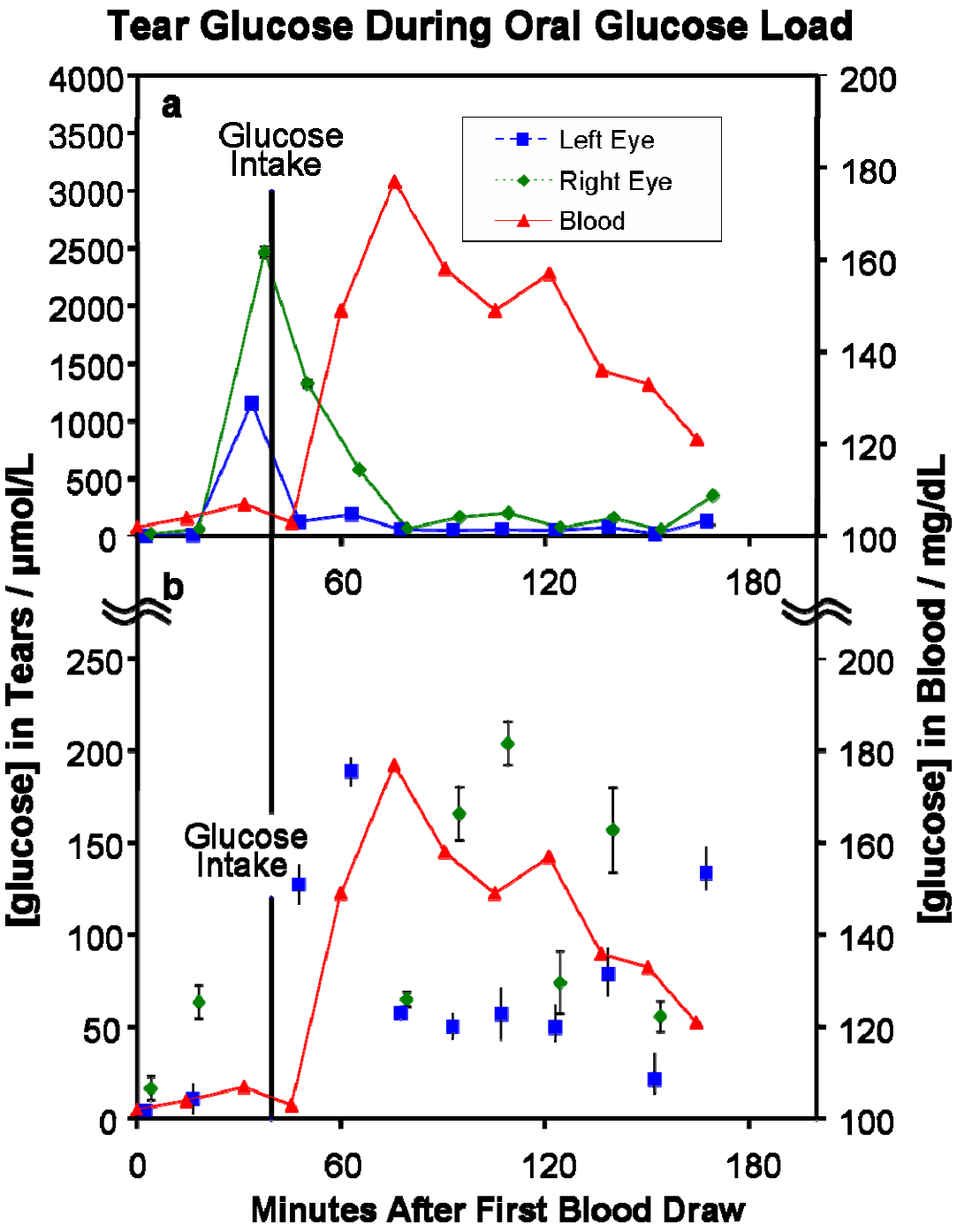

Figure 4.4. Tear and blood glucose concentrations in a replicate of the study shown in Figure 4.2. a) The tear glucose concentration scale must be expanded to show the large and abrupt increase in tear glucose before glucose intake. Blood glucose concentration peaks at ~40 minutes after glucose ingestion with a ~80% increase. b) Plotting the tear glucose on the same scale as in Figure 4.2 highlights that the tear glucose concentration appears to track the blood glucose concentration except for the early spike in tear glucose concentrations.

Identical sampling and tear glucose determination procedures were followed, and the same glucose load was given. Prior to glucose ingestion the blood glucose concentrations are similar for both experiments (~105 mg/dL). Basal tear glucose concentrations at the earliest times are also similar (~20 μmol/L). In contrast to the first study, we see an abrupt increase in the tear glucose concentration before glucose intake. The right eye tear glucose concentration increases by ~100 fold, while the left eye tear glucose concentration increases by ~50 fold, suggesting non-physiologic transport of glucose into the tear fluid. The timing and magnitude of this spike in tear glucose concentration suggest that the increase may be due to a perturbation during sampling. While we attempted to exclude this possibility, the increased glucose concentration in both eyes might suggest that the subject rubbed his eyes after slight conjunctival irritation.

The tear glucose concentrations appear to return to baseline after about 60 minutes. This time scale agrees with the time that Daum and Hill measured for tear glucose to return to baseline after the conjunctiva was stimulated by a cotton applicator.[82] When the results of the replicate study are plotted on the same scale as Figure 4.2, we see that the tear glucose concentrations at latter times (after 60 minutes) appear to track changes in the blood glucose concentration. However, the relative tear glucose elevation (~5-fold) in the left eye is much less than observed in Figure 4.2, whereas the relative increase in tear glucose concentration in the right eye is comparable.

The bulk of our studies show that before glucose intake, the blood and tear glucose concentrations remain relatively constant. Repeat measurements of tear glucose concentration in the presence of a constant blood glucose concentration can be used to define the baseline relationship between tear and blood glucose. After glucose ingestion, the blood and tear glucose

concentrations generally increase together, with an apparent 20-30 minute delay between increases in blood glucose concentration and in tear glucose concentration.

In our other studies of tear glucose concentration in subjects undergoing a glucose tolerance test, we see only a few instances of the 50-100 fold spikes in tear glucose concentration seen in figure 4. In general, these abrupt changes do not seem to correlate between the different eyes of the same subject. In future studies it would be desirable to use a hyperglycemic clamp[96] to maintain blood glucose at a constant, elevated concentration while tear glucose is measured repeatedly. This would clarify the relationship between tear and blood glucose concentrations in the presence of elevated blood glucose concentration.

While most studies of tear and blood glucose correlation collect and analyze tears from a single eye, we measured glucose concentrations in tears from both eyes. Our preliminary results suggest that tear glucose may differ between the left and right eye of a single subject. While we previously showed a general correlation between the tear glucose in the right and left eyes of fasting subjects,[30] we occasionally observe significant differences in glucose concentration between eyes. Future studies that analyze tear glucose in both eyes could help to better define the correlation in tear glucose concentration between left and right eyes.

While we do not have enough data to specify the precise relationship between tear and blood glucose concentrations over time and its variation between subjects, we believe that our tear collection method, which specifically attempts to avoid mechanical stimulation of the conjunctiva, can answer some of the outstanding questions regarding the utility of tear glucose sensing for monitoring or detecting diabetes. We have shown that we can track changes in tear and blood glucose concentrations over time. However, our results to date indicate that repeated tear sampling can effect glucose concentration; it may by extraordinarily difficult to completely

and accurately characterize daily tear glucose dynamics with a method that requires the collection of a tear sample.

4.4 *IN SITU* TEAR GLUCOSE MEASUREMENTS

There are limited reports of *in situ* tear glucose determinations using contact lens-based devices. March *et al.* developed and reported the first clinical trial of a contact lens tear glucose sensor.[24] This sensor uses fluorescence to report on the tear glucose concentration using a competitive binding mechanism. As the glucose concentration increases in the modified contact lens, quenching groups are displaced and the fluorescent signal increases. In this study, the absolute fluorescent signal was not calibrated to allow the determination of absolute glucose concentration. Rather, fluorescence intensity changes reported on relative changes in tear glucose concentrations. This group also developed a hand held photofluorometer to monitor the fluorescent signal. March *et al.* reported a glucose tolerance test for five diabetic subjects wearing the sensors. While the fluorescent signal appeared to track the blood glucose concentration, the scale had to be changed for each subject in order for the fluorescence signal to fit the blood glucose concentration profile. This study clearly showed that changes in tear and blood glucose concentration correlate. However, this approach would be unable to predict blood glucose levels in a patient without extensive calibration of the sensor response and of the correlation between tear and blood glucose concentration each time a new contact lens was inserted.

Domschke *et al.* developed a holographic, glucose-sensitive contact lens and tested it in a single subject.[25] The wavelength of light diffracted from the contact lens changed as the

holographic spacing changed in response to glucose binding. A red-shift in diffracted wavelength indicated an increase in glucose concentration. This sensor motif eliminates the challenge of measuring absolute fluorescence intensities. This sensor also detects relative changes in tear glucose concentration. Domschke *et al*. did not show a calibration curve for the diffracted wavelength dependence on glucose, and only reported the peak diffraction wavelength as a function of time. The peak diffraction wavelength appears to track the increasing blood glucose concentration with little or no delay. These results, while clearly preliminary, indicate the potential utility of monitoring tear glucose concentrations in order to determine blood glucose concentrations. Larger studies are needed to evaluate the possibility of long-term tear glucose monitoring with these contact lens sensors.

4.5 THE FUTURE OF TEAR GLUCOSE SENSING

Any future studies that measure *ex vivo* tear glucose concentrations must use non-stimulating or minimally stimulating collection methods. As reviewed here, the literature clearly demonstrates spuriously high tear glucose determinations when using paper strip collection methods that contact the conjunctiva. While it is clear that measured tear glucose concentration can vary throughout the day[28] and from eye to eye[30] it is still not clear to what extent these variations are biological and to what extent they are introduced by the sampling method. Further studies of conjunctiva and lacrimal gland immunohistochemistry along with the cell signaling mechanisms behind glucose transport in these tissues may help to better discriminate between biological and sampling variations.

While several large studies have found evidence for a correlation between averaged tear and blood glucose concentrations, the nature of this correlation within individuals is not yet fully characterized. The limited studies of critically ill patients, which showed little blood-tear glucose correlation within individuals, contrasts with the clinical studies of contact lens-based glucose sensors, which showed significant correlations between blood and tear glucose concentrations.

There is clearly enough evidence of a correlation between tear and blood glucose to justify continued efforts to develop contact lens glucose sensors. Due to the difficulties of collecting tear samples without altering the glucose concentration, it may be difficult to predict how well these sensors will work on the basis of *ex vivo* glucose analysis alone. Only well designed *in vivo* animal studies or further clinical trials of contact lens sensors in humans will be able to determine the potential utility of glucose sensing contact lens sensors to help achieve glycemic control though non-invasive glucose monitoring.

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4.5.2 Financial Disclosures

Sanford A. Asher is the scientific founder of Glucose Sensing Technologies LLC, a company developing PCCA sensors for glucose sensing.

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5.0 POLYMERIZED CRYSTALLINE COLLOIDAL ARRAY SENSORS FOR POINT-OF-CARE DETECTION OF MYOCARDIAL ISCHEMIA

Baca JT, Finegold DN, Asher SA. Clinica Chimica Acta 2007; Submitted

5.1 INTRODUCTION

Cardiovascular disease is the leading cause of death in the United States, and approximately 1.2 million Americans will have a coronary attack this year.[1] One of the challenges of treating Acute Coronary Syndrome (ACS) is the difficulty in rapidly and definitively diagnosing the cause of chest pain; only 10% to 15% of patient presenting with chest pain actually have myodardial infarction.[2] The cost of unnecessary admissions to US hospitals for patients with suspected (ACS) is ~12 billion dollars annually.[2]

Beyond patient history, the electrocardiogram (ECG) is the initial test used to evaluate patients with chest pain.[3,4] The initial ECG, however is non-diagnostic in 45% of cases of acute myocardial infarction[5] and appears completely normal in 20% of cases.[6] Blood markers such as troponins can provide a definitive diagnosis. However, they do not appear in the blood for 3-4 hrs after a myocardial infarction, after irreversible cardiac damage has occurred.[7] A point-of-care clinical chemistry test that quickly detects myocardial ischemia would help expedite treatment and avoid unnecessary hospital admissions.[8]

A decrease in serum binding affinity for Co (II) after ischemia has been described in recent reports and this affinity change can potentially be used to detect ischemia before infarction occurs.[9,10] This change in binding affinity has been termed "Ischemia Modified Albumin" by some investigators, although it is possible that albumin modification is not the deterministic phenomenon causing changes in Co (II) affinity during ischemia.[11] The implication of Human Serum Albumin (HSA) as the origin of the affinity change results because HSA is the most abundant metal binding site in human serum; the N-terminus of HSA contains a high-affinity (Ka ~ 10-15) binding site for Co (II), Cu (II), or Ni (II).[12,13]

After an ischemic event, the affinity of human serum for these metals is decreased.[14,15] The observed decrease in cobalt binding capacity is due either to the competitive occupation of metal binding sites by Cu (II) or degradation of the site by reactive oxygen species, whose formation is catalyzed by copper.[16,17] It should be noted that upon an ischemic event, anaerobic metabolism produces lactic acid, lowering the local pH and leading to the local release of copper and iron complexes.[18] Increases in serum nickel levels of ~20 nmol/L are also observed after cardiac ischemia.[19]

The decreased cobalt (II) binding affinity disappears 6 hours after transient, induced myocardial ischemia,[14] suggesting that competitive binding of locally released copper (II) is the major factor responsible for decreased cobalt (II) binding. Studies of commercial albumin preparations incubated at physiological temperatures for several weeks suggest that the N-terminal albumin binding site for transition metals may also be degraded by a metal-independent, internal cyclization mechanism.[17] This reaction appears to be too slow to account for the rapid decrease in cobalt (II) affinity observed after transient myocardial ischemia.

A reduction in the cobalt binding capacity of human serum has been measured in several clinical trials, and was shown to be useful in ruling out myocardial ischemia, especially when combined with other tests.[11,20,21] The cobalt binding assay involves complex sample preparation and access to a central clinical chemistry laboratory.[22] Methods that could detect a change in the cobalt (II), nickel (II), or copper (II) binding capacity of human serum or plasma at the bedside would improve the utility of this test as an early indicator of ischemia. A point of care assay that does not require specialized instrumentation and sample handling could dramatically improve care for millions of patients presenting with ACS.

Polymerized crystalline colloidal array (PCCA) sensors developed by Asher et al. have great potential for point of care sensing applications.[23-25] PCCAs contain a highly ordered array of colloidal nanoparticles embedded in a hydrogel matrix.[26] In low ionic strength solutions, a crystalline colloidal array (CCA) forms when highly charged polystyrene spheres self-assemble into a face centered cubic lattice. The spacing between lattice planes can be selected so that the CCA Bragg-diffracts light in the visible range. A PCCA is formed when the CCA is entrapped in a hydrogel such as polyacrylamide by photopolymerization. The lattice spacing and the resulting diffracted wavelength becomes a function of hydrogel volume, as swelling or shrinking causes a change in the lattice plane spacing. PCCA sensors can be formed by modifying the hydrogel so that a volume change occurs in response to interaction with a specific analyte. At the high ionic strength conditions found in human blood, the most straightforward approach is to design the PCCA sensor so that hydrogel crosslinks are formed or broken in response to the analyte. For example, Alexeev *et al*. recently demonstrated a PCCA sensor that responds to glucose when boronic acid groups attached to the PCCA hydrogel bind glucose at two sites of the molecule.[27]

We present here a PCCA sensor that responds to Ni (II) ions at physiological pH and osmolarity. We show that the sensor responds to Ni (II) in the presence of human plasma, and that it can be used to measure the Ni (II) binding capacity of the constituents of human plasma. We previously demonstrated PCCA sensors that respond to metal ions such as Co (II), Ni (II), and Cu (II) in aqueous solutions at low pH.[28] In the work here, we specifically study Ni (II) sensing at neutral pH. Ni (II) is more soluble that Cu (II) at neutral pH and it does not undergo the M (II) → M (III) oxidation that complicates Co ion sensing.[28] The PCCA metal sensors respond rapidly (within minutes) to changes in Ni (II) concentration, and this method of detecting a change in metal binding affinity for plasma is potentially faster than the cobalt binding assay. Since the PCCA metal sensors respond in human plasma, it is not necessary to wait for clot formation and to centrifuge the blood sample before analysis. Red blood cells can be rapidly separated from the plasma with size selective membranes or microfluidic techniques.[29,30] The sensor presented here could potentially shorten the analysis time for changes in the metal ion affinity of serum or plasma from ~60 minutes to 5-10 minutes.

5.2 METHODS

Monodisperse, highly charged polystyrene nanoparticles were prepared as previously described.[31] The particle diameter was measured to be 189 ± 9 nm (mean ± SD) by TEM. After dialysis against deionized water and mixing with AG 501-X8 (D) ion-exchange resin (20 to 50 mesh, mixed bed, Bio-Rad), the system self-assembled into a CCA and exhibited a primary diffraction peak at 562 nm at normal incidence.

PCCA were formed by photopolymerization of acrylamide (0.10 g, 1.4 mmol, Sigma) and N,N′-methylenebisacrylamide (0.002g, 13 μmol, Fluka) dissolved in the CCA (20% w/w dispersion of polystyrene latex spheres) following the procedures of Asher et al.[28] Before polymerization, the colloidal suspension and prepolymer solution was degassed, and the evacuation chamber was backfilled with nitrogen. This solution was injected into a polymerization cell (Figure 5.1) that was evacuated in order to remove oxygen. A gel support film containing surface vinyl groups (Bio-rad) used as one face of the polymerization cell, covalently attached to the PCCA during photopolymerization (12 min with two 100 watt UV lamps, Black Ray). After polymerization the PCCA was removed from the polymerization cell, cut into 10 pieces and stored in deionized water.

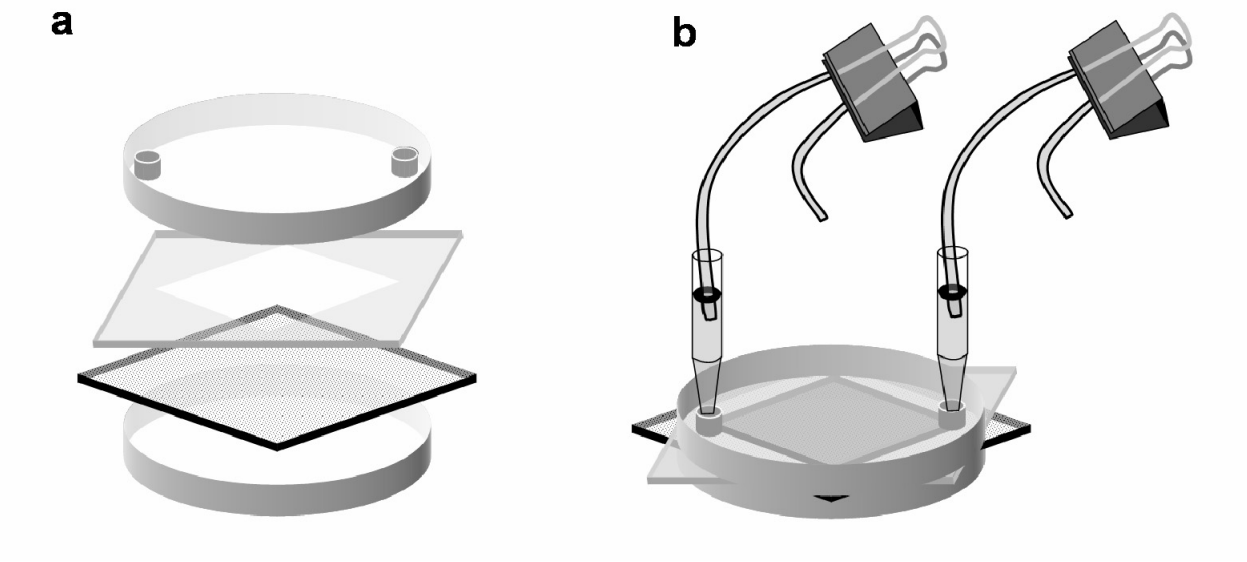

Figure 5.1. Polymerization flow cell (a) consists of a quartz plate with access holes, a parafilm spacer, a gel support film, and a solid quartz plate (top to bottom). The degassed CCA prepolymer suspension is injected through tubing connectors (b) and sealed from the environment in order to exclude oxygen during polymerization.

Metal ion sensors were made from the PCCA by the method of Asher et al.[28] The PCCA was first hydrolyzed at room temperature for 2 hours in an aqueous NaOH solution (30 mL, 0.1 mol/L, Sigma) with 10% v/v N,N,N,N-tetramethylethylenediamine (TEMED; Aldrich). The

hydrolyzed PCCA was then placed in a 30 mL aqueous solution of 5-amino-8-hydroxyquinoline (0.14 g, 0.60 mmol, Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; 0.14 g, 0.73 mmol, Pierce) for 2 hours. After rinsing with deionized water, this coupling process was repeated once again.

$NiCl_2$ stock solutions were prepared by dissolving $NiCl_2 \cdot 6H_20$ (1.189 g, 5.00 mmol, Sigma) in either 100 mL of sodium acetate (50 mmol/L, Fisher) buffered saline or 100 mL of N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES; 50 mmol/L, Sigma) buffered saline. The pH was adjusted to 4.2 for the acetate buffered solution and to 7.4 for the HEPES buffered solution by addition of 20% NaOH (Sigma). Solutions with lower Ni (II) concentration were prepared by serial dilution.

Before measuring the response of the PCCA sensors to Ni (II), they were first exposed to 50 mmol/L $NiCl_2$ solutions for at least 30 minutes, followed by rinsing with the appropriate buffer solution. This was to ensure that the sensors operated in the reversible domain, where the diffracted peak wavelength red-shifts with increasing metal ion concentration.[28] When a metal ion PCCA sensor is first exposed to low concentrations (0 – 10 μmol/L) of divalent ions such as Ni (II), Co (II), or Cu (II), there is an initial blue-shift of the peak diffracted wavelength as each ion complexes to two 8-hydroxyquinoline moieties to form hydrogel crosslinks. At higher concentrations (10 μmol/L – 50 mmol/L), these crosslinks are broken and the diffracted wavelength red-shifts as metal ions are bound by single 8-hydroxyquinoline groups. The sensing response in the red-shifting domain is reversible, whereas the sensor acts as a dosimeter in the initial blue-shifting domain.[28]

For initial studies of the sensor response, the analyte solutions were exchanged before each diffraction peak measurement in order to vary the metal ion concentration. We maintained

a volume ratio of approximately 1000:1 for the solution volume to the PCCA sensor volume. We measured replicates of the peak diffraction wavelength of a PCCA sensor at different Ni (II) concentrations in order to determine the standard deviation of the measurement.

Standard addition studies were conducted by adding small volumes (50-500 μL) of concentrated Ni (II) solution to the large (30-50 mL) buffer reservoir bathing the sensor. The diffraction of the PCCA was monitored using a 6 around 1 reflectance probe and a fiber-optic diode spectrometer with a tungsten halogen light source (Ocean Optics).

For PCCA dehydration experiments, the sensor was left in a HEPES buffered saline solution containing a total of ~1.5 mmol $NiCl_2$ and allowed to dehydrate by exposure to room air over 3 days. The sensors were rehydrated by adding deionized water to the dish, and rinsed with HEPES buffer prior to additional sensing experiments.

For the Ni (II) binding experiments, 10 mL of human plasma was added to 20 mL of HEPES buffered saline solutions with known Ni (II) concentrations. This human plasma was separated from a pooled, heparinized whole blood sample (Bioreclamation) by centrifugation. The sodium heparin had a molecular weight of ~12 kDa and was present at a concentration of 15 USP units per 1 ml of whole blood.

5.3 RESULTS

Figure 5.2 shows several sequential measurements of the peak diffracted wavelength for a PCCA sensor as the Ni (II) concentration is varied. Based on the variance of these measurements, we calculate a maximum standard deviation of 0.6 nm for the peak diffracted wavelength at the different Ni (II) concentrations and a standard deviation of 0.2 nm at 0.75 mmol/L. The peak

diffracted wavelength shift is roughly linear over Ni (II) concentrations between 0.2 mmol/L and 1.0 mmol/L (Figure 5.3a), which is the relevant pathophysiologic range for monitoring changes in plasma metal binding capacity. We can use the slope of the response curve (8.6 nm/(mmol/L)) to express the standard deviation in terms of Ni (II) concentration (0.07 mmol/L at 7.5 mmol/L and 0.02 mmol/L at 0.75 mmol/L).

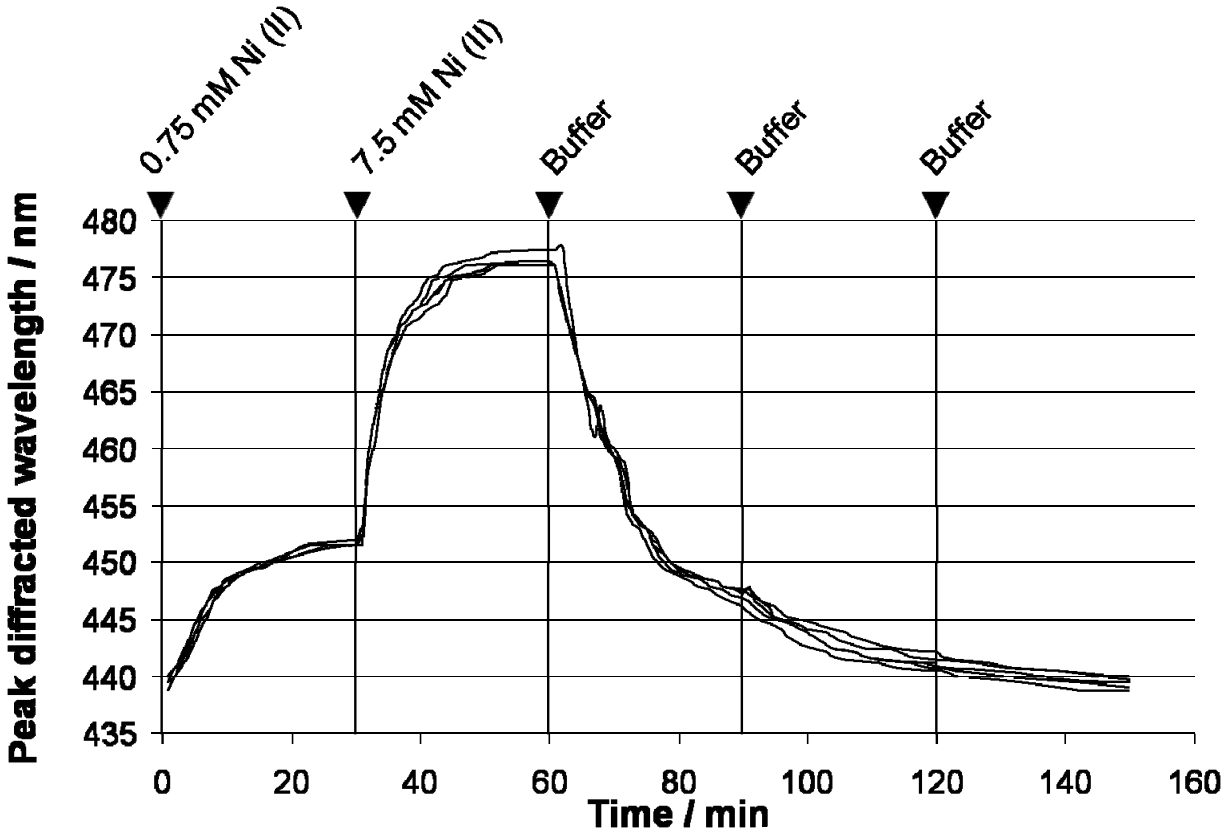

Figure 5.2. Time dependence of peak diffracted wavelength of the PCCA sensor upon alteration of Ni (II) concentration for 5 consecutive replicates. Solutions contained 50 mmol/L HEPES buffer and 150 mmol/L NaCl. Each curve represents a different replicate cycle. Vertical lines indicate times when the analyte solution was replaced. Added solutions contained 0.75 mmol/L $NiCl_2$ at 0 minutes and 7.5 mmol/L $NiCl_2$ at 30 minutes. At 60, 90, and 120 minutes, the solution was replaced with HEPES buffered saline. The peak diffracted wavelength was 439.5 ± 0.5 nm at 0 mmol/L NiCl2, 451.7 ± 0.2 nm at 0.75 mmol/L, and 476.5 ± 0.6 at 7.5 mmol/L (average ± SD). This yields an overall standard deviation of ± 0.5 nm.

Figure 5.3 shows the response of a PCCA Ni (II) sensor at pH 4.2, and pH 7.4. The response profiles are very similar, except that diffraction is blue-shifted by approximately 10 nm at the higher pH.

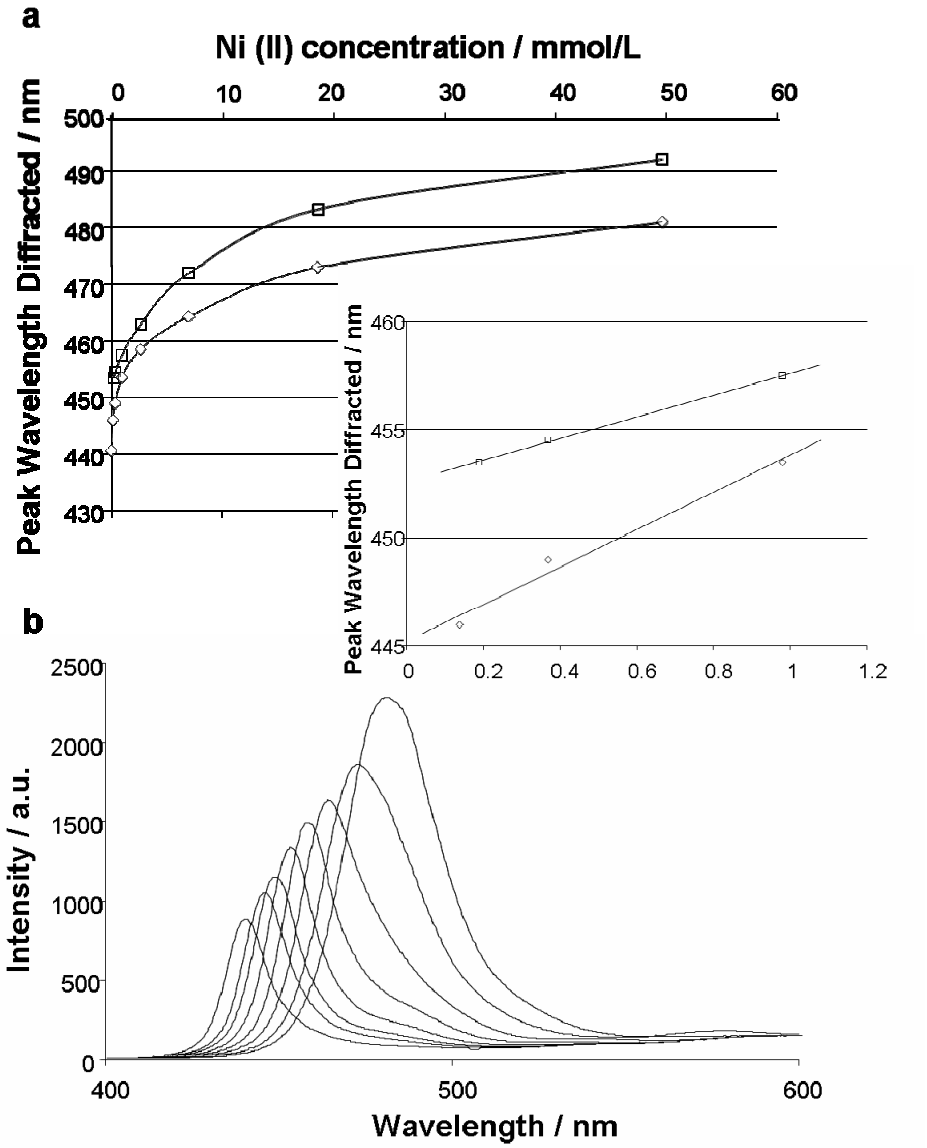

Figure 5.3. a) The dependence of peak diffracted wavelength on $NiCl_2$ concentration and pH: pH=4.2 (□) and pH=7.4 (◊). At pH=4.2, solutions contain 50 mmol/L sodium acetate and 150 mmol/L NaCl. At pH=7.4, solutions contain 50 mmol/L HEPES buffer and 150 mmol/L NaCl. The inset shows the linear response range between 0.2 mmol/L and 1.0 mmol/L Ni (II). b) Observed reflectance diffraction spectra for the pH=7.4 curve points.

Figure 5.4 shows the effects of dehydrating the PCCA sensor. The response profiles before and after dehydration are very similar, except that peak diffraction red-shifts and the sensor sensitivity decreases. For example, the wavelength shift in response to 1 mmol/L Ni (II) was diminished from 12.9 nm to 8.8 nm. The intense Bragg diffraction peak that is indicative of PCCA ordering is recovered after rehydration (Figure 5.4 inset).

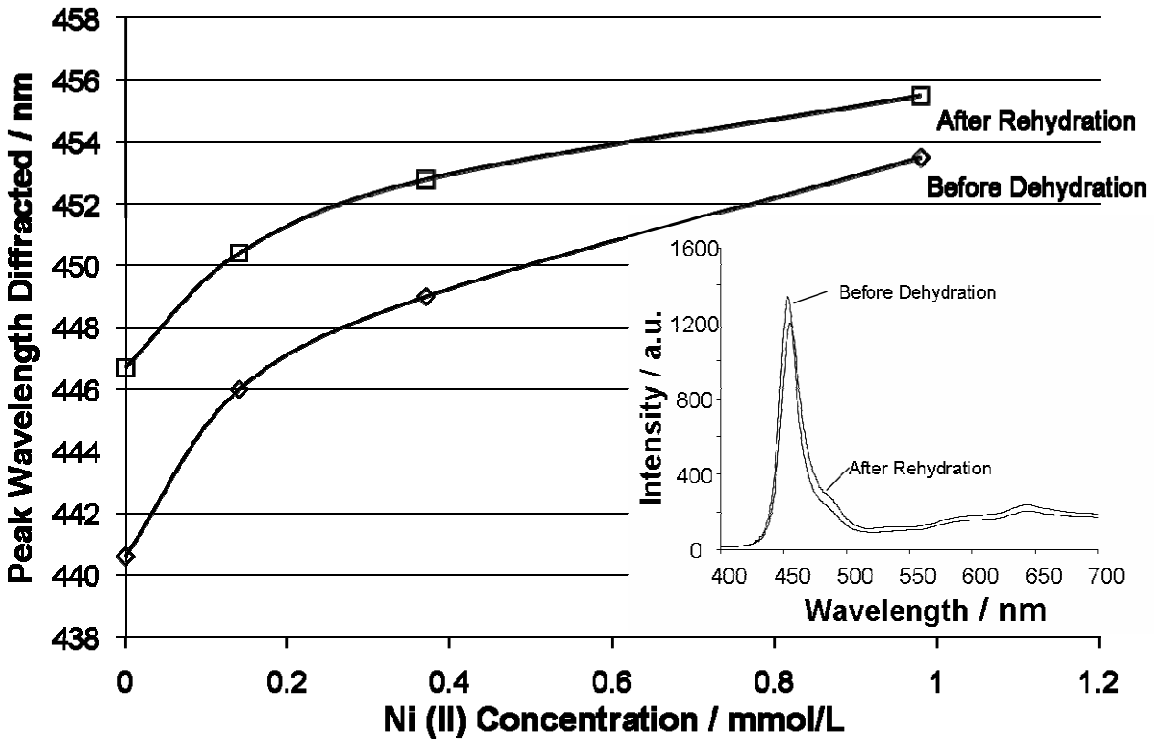

Figure 5.4. Dependence of peak diffracted wavelength on $NiCl_2$ concentration before (◊) and after (□) dehydration. The sensitivity of the PCCA sensor is decreased after rehydration as the peak wavelength diffraction shift between 0 to 1 mmol/L $NiCl_2$ decreases from 12.9 nm before dehydration to 8.8 nm after rehydration. The inset shows the diffraction spectrum at 0.98 mmol/L before and after dehydration. The peak diffracted wavelength shifts 2 nm from λ=453.5 nm (before dehydration) to λ=455.5 nm (after rehydration). The PCCA sensor was dehydrated and rehydrated *in situ*. Thus, the diffraction spectra are from identical spots of the sensor.

Addition of human plasma to a nickel-free buffer solution surrounding the sensor shifts the peak diffracted wavelength by ~2 nm (Figure 5.5). Initially, there is a small, rapid blue shift, followed by a much smaller and more gradual red-shift. After the removal of plasma, and

rinsing with buffer, the peak diffracted wavelength returns to the original value. We observe no evidence of coagulation or clot formation during these experiments.

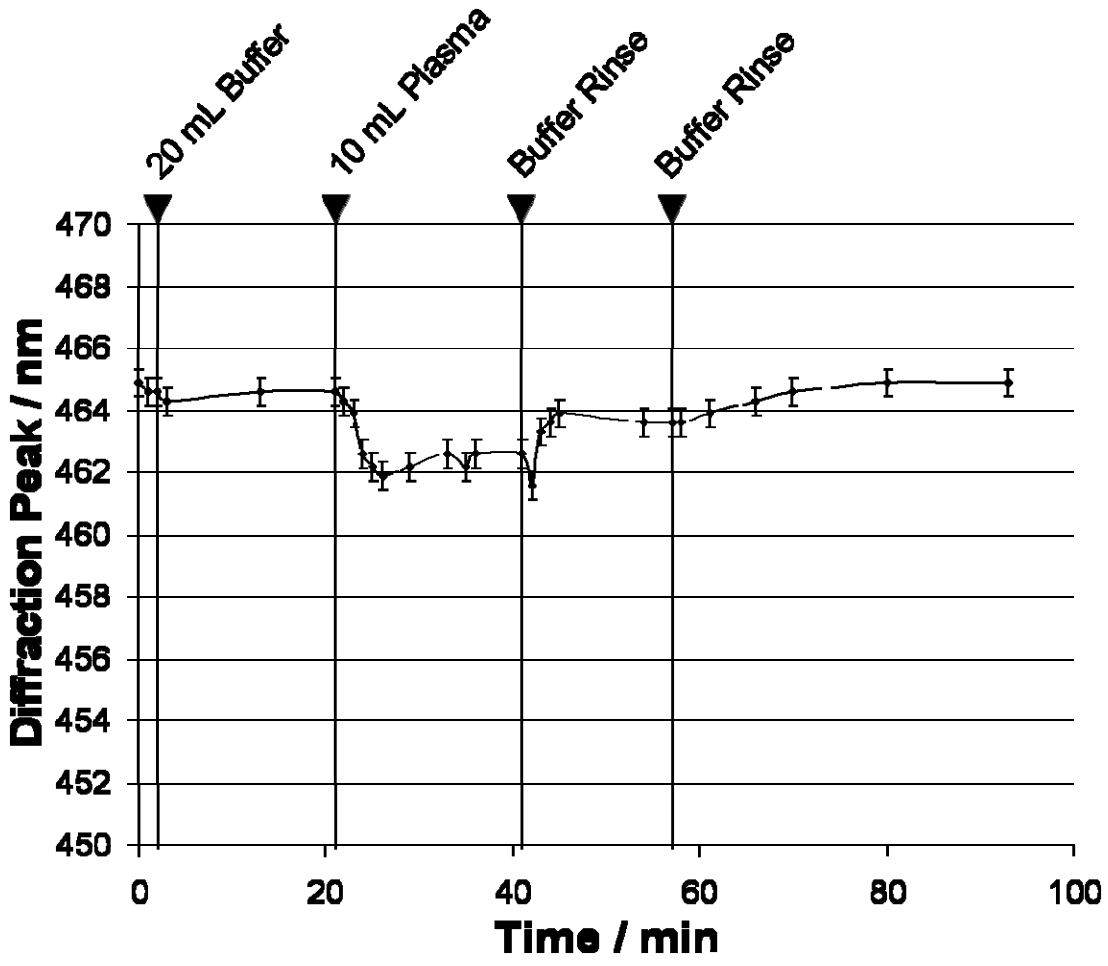

Figure 5.5. (a): Response of PCCA sensor to human plasma in the absence of $NiCl_2$. Plasma was added to the analyte solution (50 mmol/L HEPES and 150 mmol/L NaCl) at 21 min to form a 2:1 (buffer:plasma) solution. After rinsing with HEPES buffer, the peak diffracted wavelength returns to its original value.

Figure 5.6a shows the effect of adding 10 mL of human plasma to a PCCA sensor equilibrated in 20 mL of a 0.75 mmol/L $NiCl_2$ solution containing 50 mmol/L HEPES buffer and 150 mmol/L NaCl. There is a rapid 12.4 nm blue-shift, which is much larger than in the absence of Ni (II). Figure 5.6b shows the effect of adding the same volume of buffer rather than plasma.

Accounting for the osmotic effects of plasma addition, we find that the human plasma binds 1.0 mmol Ni (II) per L of human plasma (see Discussion below).

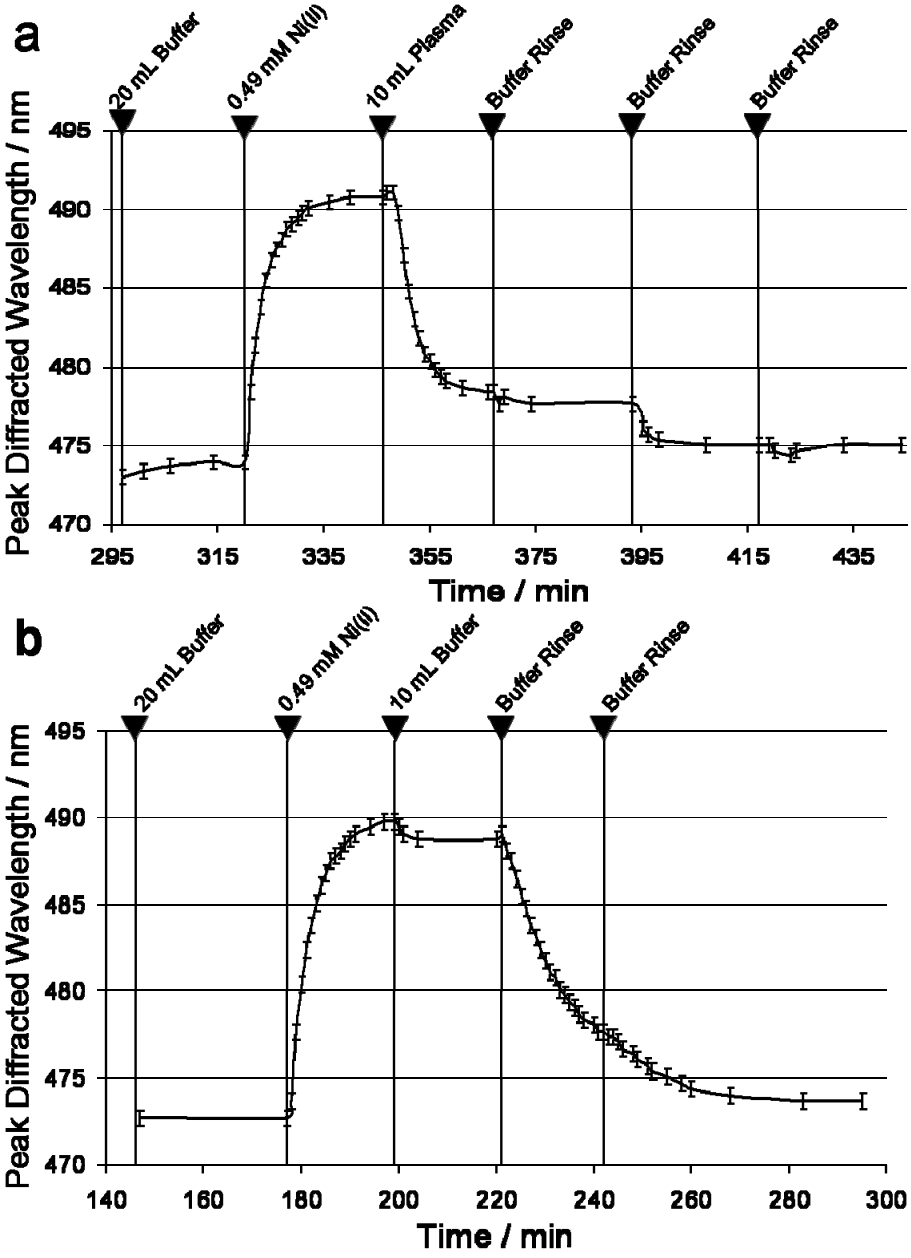

Figure 5.6. (a) Response of PCCA sensor to human plasma addition in the presence of $NiCl_2$. The sensor initially red-shifted in response to 0.74 mmol/L aqueous $NiCl_2$ in 50 mmol/L HEPES buffer and 150 mmol/L NaCl. Addition of human plasma at 346 minutes caused a blue-shift of 12.4 nm. (b) Response of PCCA sensor to HEPES buffer addition in the presence of $NiCl_2$. Addition of HEPES buffer at 199 min caused a blue-shift of 0.7 nm, due to dilution of $NiCl_2$ to a concentration of 0.49 mmol/L.

We observe a sustained and linear red-shift of 0.5 nm/hr after the addition of plasma and Ni (II) (not shown). This small red shift is within the error of the measurement for a single concentration determination lasting 10-20 minutes.

5.4 DISCUSSION

Based on the standard deviation of 0.02 mmol/L (at a Ni (II) concentration of 0.75), we can expect to reliably detect changes in plasma binding capacity of 60 μmol/L. PCCA metal sensors were previously studied at pH 4.2.[28] However, the affinity of the N-terminal metal binding site in HSA for Co (II) markedly decreases at pH values below 6.2.[15] The response profiles at pH 4.2 and pH 7.4 are very similar, except that diffraction is blue-shifted by approximately 10 nm at the higher pH (Figure 5.3). This difference is likely due to a slightly lower affinity of 8-hydroxyquinolines for Ni (II) at lower pH.[32]

The ability to maintain PCCA sensors in dry storage before use would greatly aid the development of these sensors for practical clinical applications. However, until now, there has been no demonstration that PCCA sensors can be reversibly dehydrated and rehydrated. Figure 5.4 shows that our Ni (II) PCCA sensor can be reversibly dehydrated. We believe this to be the first report of a PCCA sensor that can be reversibly dehydrated and rehydrated. (Sanford Asher, Michelle Muscatello and Lee Stunja in our lab are studying the reversible dehydration of PCCA glucose sensors, and are in the process of preparing a manuscript on this topic.) Unconstrained PCCA sensors generally become brittle during the drying process and often crack. Attachment to a gel support film seems to force the sensor to dry more uniformly, which prevents formation of cracks. Polyacrylamide electrophoresis gels are often dried in the presence of concentrated,

low molecular weight stabilizers such as glycerol, urea, or ethanol[33,34]; the presence of the HEPES buffer during drying likely acts in a manner similar to other low molecular weight stabilizers, preventing the irreversible collapse of polyacrylamide chains.

While the response of Pb (II), creatinine, and ammonia PCCA sensors in human and bovine serum has been studied previously,[23,24,35] the response of PCCA sensors to human plasma has not been investigated. As shown in Figure 5.5, the sensor exhibits a small and rapid initial blue-shift followed by a smaller and more gradual red-shift. This two-phase response is typical of hydrogels placed in a hypertonic solution evolving to osmotic equilibrium.[36] The addition of plasma initially creates hypertonic conditions outside of the hydrogel and water flows out of the hydrogel to equalize the osmotic pressure. At longer time scales, plasma proteins such as albumin diffuse into the hydrogel, diminishing the osmotic pressure. Previous work suggests that the diffusive permeability of albumin in a polyacrylamide hydrogel such as those studied here is approximately 10^{-8} cm^2s^{-1}.[37] This means that on the time scale of the experiment (20 minutes), the average albumin molecule will diffuse only about 50 μm into the hydrogel. For this reason, the red-shifting back towards the baseline wavelength is slow.

The large PCCA sensor blue shift in response to plasma, when Ni (II) is present, cannot be explained by osmotic effects of plasma or by dilution effects. Figure 5.6b shows the effect of adding the same volume of buffer rather than plasma. We hypothesize that the components proteins of human plasma, especially albumin, sequester Ni (II), leading to a blue-shift in the diffracted wavelength as the effective concentration of Ni (II) decreases. Comparing the diffracted wavelength shifts for our sensor after plasma addition with the Ni (II) calibration curve allows us calculate the concentration of Ni (II) bound. Accounting for the osmotic effects of plasma addition described above, we find that the human plasma preparation binds 1.0 mmol Ni

(II) per L of human plasma. The reference interval for human serum albumin in adults is 35-52 g/L (0.53-0.78 mmol/L). Hence, other species in the plasma preparation must also have a significant affinity for Ni (II). Nickeloplasmin[38] and histidine[39] have been identified as the major species besides serum albumin that bind nickel in human plasma. Proteins involved in the clotting cascade[40] (factor VIII) and the alternative complement pathway[41] (C-3 convertase) are also known to bind Ni (II). While such species will decrease the total free Ni (II) concentration measured by our sensors, unless their Ni (II) binding affinity changes due to ischemia, they will not impact the measurement of ischemia induced changes in plasma Ni (II) binding.

As noted above, we observe a small, linear red shift over time after the addition of plasma and Ni (II). One plausible explanation is that albumin or other plasma proteins are reacting with and/or binding to the hydrogel. Binding of these proteins may alter the free energy of mixing of the hydrogel. This small red shift is within the error of the measurement for a single concentration determination lasting 10-20 minutes. However, it would become significant during longer experiments, complicating standard addition experiments, where several measurements must be made after the addition of plasma.

We demonstrate here the ability to measure a change in the nickel concentration in the presence of human plasma as it is sequestered by the constituents of plasma. This is the required first step in developing a PCCA sensor for the rapid detection of myocardial ischemia. Future studies will be needed to establish reference intervals and to standardize this assay.

Previous studies suggest that it is possible to make polyacrylamide PCCA metal sensors which undergo greater wavelength shifts in response to Ni (II), Co (II), or Cu(II).[28] This is generally accomplished by modifying the hydrogel volume fraction and initial cross link density. If the sensors showed large wavelength shifts between 0 and 1 mmol/L a simple visual

inspection of the sensor could be used to determine the test result. However, since some end users may be color blind, and for documentation purposes, it may be preferable to use a handheld spectrometer to determine the peak diffracted wavelength.

5.4.1 Grant/funding support

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5.4.2 Financial Disclosures

Sanford A. Asher is the scientific founder of Glucose Sensing Technologies LLC, a company developing PCCA sensors for glucose sensing.

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6.0 SYNTHESIS OF UNIFORM PCCA SENSORS ON SOLID SUPPORT

One of the difficulties in fabricating and testing PCCA sensors is that they must be immobilized against a flat surface in order to ensure that diffraction is monitored along the normal direction. Previous solutions to this problem often rely on the fact that during some stage of the fabrication process, PCCA sensors often become "extremely sticky".[1] This allows the sensor to adhere to a polystyrene Petri dish when it touches the surface. This process can lead to wrinkles in the hydrogel surface, potentially complicating diffraction measurements. It also makes removal of the PCCA sensor from the Petri dish either difficult or impossible. The Asher group has previously reported attachment of a PCCA sensor for lead to a plastic film in order to form an optrode.[2] This approach, however, has not been widely applied to other PCCA sensors and is not described in detail in the literature.

Polyacrylamide gels are often used in biochemical studies for the separation of proteins or nucleic acids.[3] These hydrogels can be covalently bound to a solid support such as GelBond PAG film (Bio-Rad) during photopolyermization. This type of solid support generally consists of a transparent plastic film coated with a resin that contains vinyl groups, which can co-polymerize with the acrylamide gel.[4]

We investigated the formation of a PCCA while the CCA precursor was in contact with a gel support film in order to covalently attach the PCCA to this gel support film. We successfully attached polyacrylamide PCCAs to GelBond gel support films, and have described the use of

these sensors in a paper submitted to Clinica Chimica Acta (Chapter 5). The present chapter expands on the synthesis of uniform PCCA sensors attached to a solid support.

6.1 EXPERIMENTAL METHODS

As described in section 5.2, PCCA were formed by photopolymerization of acrylamide (0.10 g, 1.4 mmol, Sigma) and N,N′-methylenebisacrylamide (0.002g, 13 μmol, Fluka) dissolved in the CCA (20% w/w dispersion of polystyrene latex spheres) following the procedures of Asher *et al.*[1] Before polymerization, the colloidal suspension and prepolymer solution was degassed and the evacuation chamber was backfilled with nitrogen. This solution was then immediately injected into a polymerization cell that had been evacuated in order to remove oxygen (Figure 6.1).

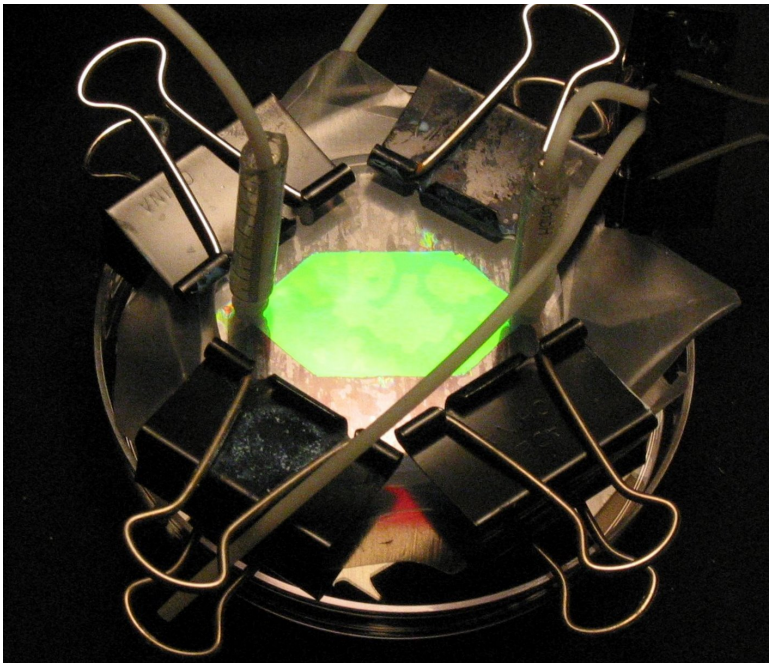

Figure 6.1. Photograph of the polymerization cell used to fabricate a PCCA attached to a gel support film. This picture was taken immediately after injection of the colloidal suspension, before polymerization. A schematic drawing of this polymerization cell appears in Figure 5.1.

The polymerization cell consists of a quartz plate on one side and a gel support film, which is supported by another quartz plate on the other side. The top quartz plate and the gel support film are separated by a parafilm spacer. In contrast to previous polymerization cells employed by the Asher group, the colloidal suspension was injected into the polymerization cell through plastic tubing connectors in order to help exclude oxygen diffusion into the cell during polymerization. In some control experiments, one side of the polymerization cell was left exposed to the atmosphere during polymerization.

The gel support film contains surface vinyl groups, which copolymerize with the PCCA during photopolymerization (12 min with two 100 watt UV lamps, Black Ray). After polymerization, the PCCA attached to the support film is easily removed from the polymerization cell, and does not adhere to the top quartz plate. The PCCA and the underlying support film can then be cut into several pieces. If a margin or "tab" is left around the PCCA when cutting the support, the PCCA can be easily manipulated and transferred without contacting it directly.

6.2 RESULTS AND DISCUSSION

Careful deoxygenation of the colloidal suspension and the polymerization cell is crucial to forming a uniform PCCA. If the suspension is not evacuated, the polymerized PCCA will often detach from the gel support film. If one side of the polymerization cell is left open to the atmosphere during photopolymerization, the resulting PCCA is not uniform (Figure 6.2).

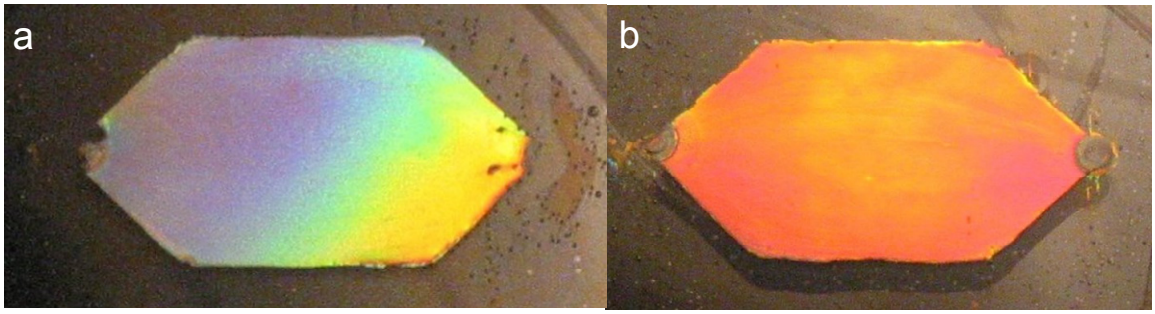

Figure 6.2. Two PCCAs attached to gel support films, and polymerized under different conditions. These PCCAs have been removed from the polymerization cell and thoroughly rinsed with deionized water. The colloidal suspensions in both cases were degassed before injection into the polymerization cell. The PCCAs were photographed immediately after polymerization, before any modification of the acrylamide hydrogel. a) When one side of the polymerization cell is left open to the environment, the diffracted wavelength is clearly not uniform. b) When the colloidal suspension is injected through plastic tubing connectors and the polymerization cell is sealed from the environment during polymerization, the wavelength diffracted by the PCCA is much more uniform.

For the PCCA shown in Figure 6.2a, the colloidal suspension was injected into the polymerization cell from left to right, and the right side remained exposed to the atmosphere during photopolymerization. The diffracted color on the right side of the PCCA is red-shifted relative to the left side. This suggests that the crosslink density of the hydrogel decreases moving from the left to right across the pictured PCCA. This change in cross link density is likely due to the presence of an oxygen gradient inside the cell during photopolymerization.

In contrast, the PCCA shown in Figure 6.2b is fairly uniform in its diffracted wavelength. The exclusion of oxygen from the polymerization cell throughout the polymerization process appears to aid in the fabrication of a uniform PCCA attached to a gel support film.

PCCAs formed in the absence of a gel support film also appear to be sensitive to oxygen gradients during polymerization (Figure 6.3).

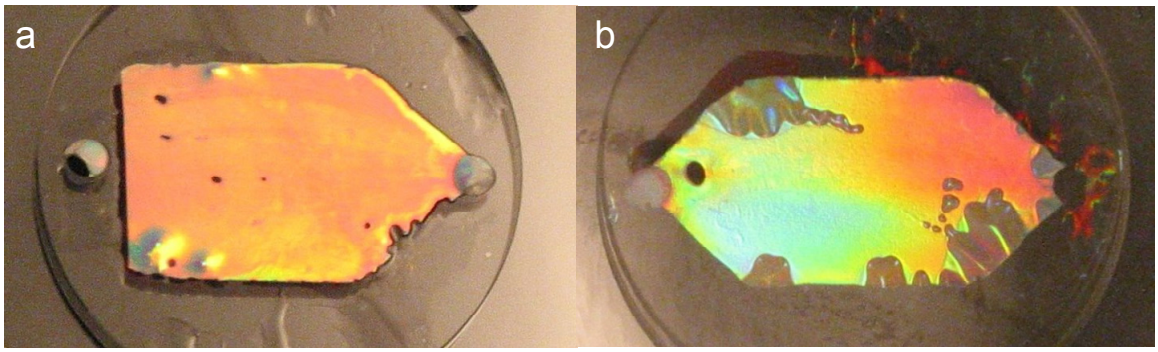

Figure 6.3. Two PCCAs that were polymerized in the absence of a gel support film. These PCCAs have been removed from the polymerization cell and thoroughly rinsed with deionized water. In both cases, the colloidal suspension was degassed before injection into the polymerization cell, and one side was left open to the atmosphere during polymerization. a) The colloidal suspension was exposed to the atmosphere for several minutes after degassing, before injection into the polymerization cell. b) The colloidal suspension was injected into the polymerization cell immediately after degassing.

These PCCAs in Figure 6.3 were formed using polymerization cells consisting of two quartz plates and a parafilm spacer; the gel support film was omitted. As in Figure 6.2a, one access hole of the cell was left open to the environment during polymerization. When the colloidal suspension is allowed to equilibrate with the atmosphere before injection into the polymerization cell, large gradients in diffracted wavelength are not observed (Figure 6.3a). However, when the colloidal suspension is immediately injected into the polymerization cell, the diffracted wavelength gradient is similar to that observed in Figure 6.2a. The colloidal suspension was injected from left to right for the PCCA in Figure 6.3b, and the right side was exposed to the environment during polymerization. Similar concentrations of acrylamide and methylenebisacrylamide were present in all colloidal suspensions prior to injection. However, the light diffracted from this PCCA in Figure 6.3b (green to red) is red-shifted relative to the light diffracted from the PCCA in Figure 6.2a (blue to yellow). The hydrogel may be particularly constrained when covalently attached to a gel support film on one side, and hence swell to a lesser extent in water.

6.3 CONCLUSIONS

We have demonstrated the synthesis of uniform PCCAs on a gel support film. This allows for easier manipulation and testing of PCCA sensors. PCCAs attached to gel support films may then be further modified to sense a variety of analytes including metal ions,[1,5] creatinine,[6] ammonia,[7] glucose,[8] and organophosphate nerve agents.[9] The method developed here can be employed in developing and testing future PCCA sensors.

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7.0 FUTURE WORK

Our investigations have contributed to the development of point-of-care sensors for use in treating diabetes and detecting myocardial ischemia. Further work is needed to complete the development of these sensors for clinical use. In this section, we outline the necessary work.

7.1 FUTURE STUDIES OF TEAR GLUCOSE

In Chapter 3, we demonstrated the utility of the tear glucose analysis method developed in Chapter 2 for measuring tear glucose concentration in fasting non-diabetic subjects. Using a sampling method designed to minimize irritation of the eye, we demonstrated that tear glucose concentration is significantly lower than previously believed. The next step will use this method in further determinations of tear glucose concentration in diabetic subjects, and in non-diabetic subjects with elevated blood glucose concentrations. We also plan in situ tests of the glucose sensors developed by Alexeev *et al.* and Ben-Moshe *et al.*[1, 2]

7.1.1 *Ex vivo* studies of tear glucose concentration

While we characterized the basal tear glucose concentration in non-diabetic subjects, it remains unclear precisely how diabetes may affect tear glucose concentrations. The microvasculature, especially in the retina, kidneys, and extremities, is often damaged due to elevated blood glucose levels in diabetic patients.[3] As described in Chapter 4, previous studies of tear glucose concentration in diabetic subjects have generally used tear collection techniques that may irritate the cornea and artificially elevate the tear glucose concentration. Further studies, using a non-stimulating collection method, are needed to characterize tear glucose concentrations in diabetic subjects. An increased tear glucose concentration in diabetic subjects could be due to an increased blood glucose concentration, or it could be due, in part, to an alteration of glucose transport in tears caused by the sequelae of diabetes.

7.1.1.1 Tear Glucose Concentration in Fasting Diabetic Subjects

We plan to study tear glucose concentration in diabetic subjects as well as in non-diabetic subjects with elevated blood glucose levels. Our previous work has shown that tear glucose concentrations can vary considerably, even within a fasting subject.[4] The standard deviation in our measurement of tear glucose for an individual is approximately half of the average tear glucose concentration for that individual.[4] We have also shown that tear glucose can increase several fold after glucose ingestion. For this reason, we will initially study tear glucose concentration in subjects where the blood glucose concentration is constant. We will collect tear glucose from diabetic subjects who have fasted overnight. While blood glucose in theses subjects may be elevated, it should remain relatively constant over a 30 minute interval. We will collect three tear samples from each eye at intervals of ~15 minutes, following the same

procedures as in our study of fasting, non-diabetic subjects.[4] This will allow us to determine the variance in tear glucose for diabetic subjects, and will help determine the relationship between tear and blood glucose in these subjects.

We previously attempted to measure tear glucose concentrations in diabetic subjects who were visiting the University of Pittsburgh Medical Center Diabetes Clinic. While this was an excellent location to recruit diabetic subjects, it was not possible to collect more than a single tear sample from each eye due to the time and space constraints of this busy clinic. Additionally, the fasting state of subjects was not always controlled. While some subjects were specifically undergoing phlebotomy for the measurement of fasting blood glucose concentration, others were having blood tests that did not require prior fasting. The tear samples from this study were analyzed using an early version of the mass spectrometry method that did not employ a column for the separation of glucose. We later determined that we could not accurately quantify glucose concentrations below ~100 μmol/L using the initial mass spectrometry method. Considering the difficulties of collecting several tear samples in a busy diabetes clinic, we plan to conduct future studies of subjects with diabetes in a research setting, where the fasting state can be controlled and where we will have time to collect at least three tear samples from each eye.

7.1.1.2 Tear Glucose Concentration in Hyperglycemic Non-Diabetic Subjects

In order to understand how elevated blood glucose concentrations affect tear glucose concentrations in the absence of diabetes, we will also study non-diabetic subjects with elevated blood glucose concentrations. We will attain constant, elevated blood glucose concentrations for these subjects by using a hyperglycemic clamp.[5] This procedure uses intravenous injection of glucose along with frequent monitoring of blood glucose concentration to maintain constant, elevated glucose concentrations. The elevated blood glucose concentrations safely attainable

with a hyperglycemic clamp in non-diabetic subjects will not be as high as the blood glucose concentrations frequently seen in diabetic subjects. However, even a modest increase in blood glucose concentration (~50%) will allow us to determine basal tear glucose concentrations in non-diabetic subjects over a much greater glycemic range. Again, we will follow the tear collection and analysis methods described in Chapters 2 and 3 so that we can compare our future and prior results.

We may need to study fairly large populations in order to obtain statistically significant measurements of tear glucose concentration. We cannot precisely predict the standard deviation of measured tear glucose concentration for hyperglycemic subjects. Our initial assumption will be that the relationship between the standard deviation and the average tear glucose concentration is the same for all subjects. An initial, pilot study of ~10 fasting diabetic subjects and ~10 non-diabetic subjects undergoing a hyperglycemic clamp will be done to help calculate the size of the population needed to obtain statistically significant results.

7.1.2 *In Situ* Studies of Tear Glucose Concentration

The most definitive way to assess the utility of monitoring tear glucose concentration with contact lens-based sensors is to test these sensors clinically. While studies that remove tear fluid from the eye help to establish the general range of tear glucose concentrations in tear fluid, they cannot predict how well an *in situ* tear glucose sensor will predict blood glucose concentration. Initial studies should be done in animals in order to characterize the biocompatibility of these sensors.

PCCA sensors have not been previously used in living animals or humans. The biocompatibility tests needed to demonstrate the safety of theses materials are well established

and are described in the ISO (International Organization for Standardization) standard ISO-10993-1:2003, (Biological evaluation of medical devices Part 1: Evaluation and testing).[6] (Table 7.1.)

Table 7.1. Biocompatibility Tests and ISO references

Biocompatibility Test	Test method	ISO reference
cytotoxicity	Agar Diffusion: determines mammalian cytotoxic potential of leachable components in the PCCA sensor.	*ISO 10993-5:1999* (*Biological evaluation of medical devices Part 5: Tests for in vitro cytotoxicity)*
skin irritation	Monitors erythema and edema in rabbit skin after prolonged contact with the PCCA sensor.	*ISO 10993-10:2002* (*Biological evaluation of medical devices Part 10)*
ocular irritation	Quantifies ocular lesions in rabbits in response to contact with the PCCA sensor.	*ISO 10993-10:2002 (E) Annex B (Ocular Irritation Test)*
contact lens biocompatibility	Monitors response of rabbit eyes using a standard scoring system under standard contact lens wear and care conditions.	*ISO 9394:1998 (Ophthalmic optics – Contact lenses and contact lens care products – Determination of biocompatibility by ocular study with rabbit eyes)*

This ISO standard describes what tests are needed to assess the biocompatibility of a medical device, and the above table was constructed to conform to these guidelines. These studies should be completed in the order listed, as any adverse result will obviate the need for further studies. While these studies can be performed in any lab with the proper certifications, it is often more expedient to have these tests done by a facility that specializes in biocompatibility testing.

Assuming that the results of initial biocompatibility tests are favorable, we will carry out further in vivo tests of contact lens-based PCCA glucose sensors. Because contact lenses are

frequently tested in rabbits, and because there are well established models of diabetes in rabbits, initial in vivo testing will be done in these animals. The normal range of blood glucose concentration is similar in rabbits and in humans. Also, the blood glucose concentration in rabbits can be easily manipulated by glucose or insulin injections. We will monitor the diffracted wavelength of light from a PCCA glucose senor while it is in the rabbit eye. We will also measure blood glucose concentration in the rabbit to determine the correlation between diffracted wavelength and blood glucose concentration. Rabbits will be particularly useful for studying the extreme ranges of hyperglycemia and hypoglycemia that may be dangerous to produce experimentally in humans.

Finally, these sensors will need to be tested in human subjects. The lenses should first be studied in habituated contact lenses wearers. Clinical protocols such as the ones described in the previous section will be used to study the sensors under a variety of blood glucose conditions. The diffracted wavelength of light will be monitored during fasting and during a glucose tolerance test. Previous studies of in situ contact lenses have demonstrated the utility of glucose tolerance tests assessing the ability of the sensor to predict blood glucose concentrations.[7, 8]

7.2 FUTURE STUDIES OF POINT-OF-CARE SENSORS FOR MYOCARDIAL ISCHEMIA

We showed in Chapter 5 that PCCA sensors can report on the metal binding affinity of plasma. In order to further develop this sensor, we will need to optimize its response over the relevant analytical range and conduct clinical trials with blood samples from ischemic and control subjects.

7.2.1 Optimization of PCCA sensors for Nickel

There are several approaches that could potentially increase the response of the PCCA sensors to changes in the metal binding affinity of plasma. Decreasing polymer crosslink density is one approach to increasing the sensitivity of polyacrylamide PCCA sensors. The sensors described in Chapter 5 have a crosslink to monomer ratio of 1:100. Decreasing this ratio will allow for greater swelling of the hydrogel so that sensor response to Ni^{2+} concentrations between 0 and 1 mmol/L covers a larger portion of the visible spectrum.

We will also attempt to attach more 5-amino-8-hydroxyquinoline groups to the hydrogel. This can be accomplished by increasing the hydrolysis of amides in the hydrogel and repeating the EDC coupling step, which attaches 5-amino-8-hydroxyquinoline to carboxylate groups. While it is difficult to quantify the attachment of the 5-amino-8-hydroxyquinoline groups to the PCCAs, we can monitor attachment of these moieties to a colloid-free polyacrylamide hydrogel as previously demonstrated.[9]

We have shown that the current sensors can distinguish changes in free Ni^{2+} concentration as low as 60 μmol/L, and this is likely sufficient to sense clinically significant changes in the metal binding affinity of plasma.[10] However this change in Ni^{2+} concentration corresponds to a peak diffracted wavelength shift of 0.6 nm. To improve the response of the PCCA sensors, we should also investigate attaching chelating agents with a higher affinity for Ni^{2+} ions. Figure 7.1 shows the response of these PCCA metal sensors to a wide range of Cu^{2+} ion concentrations.

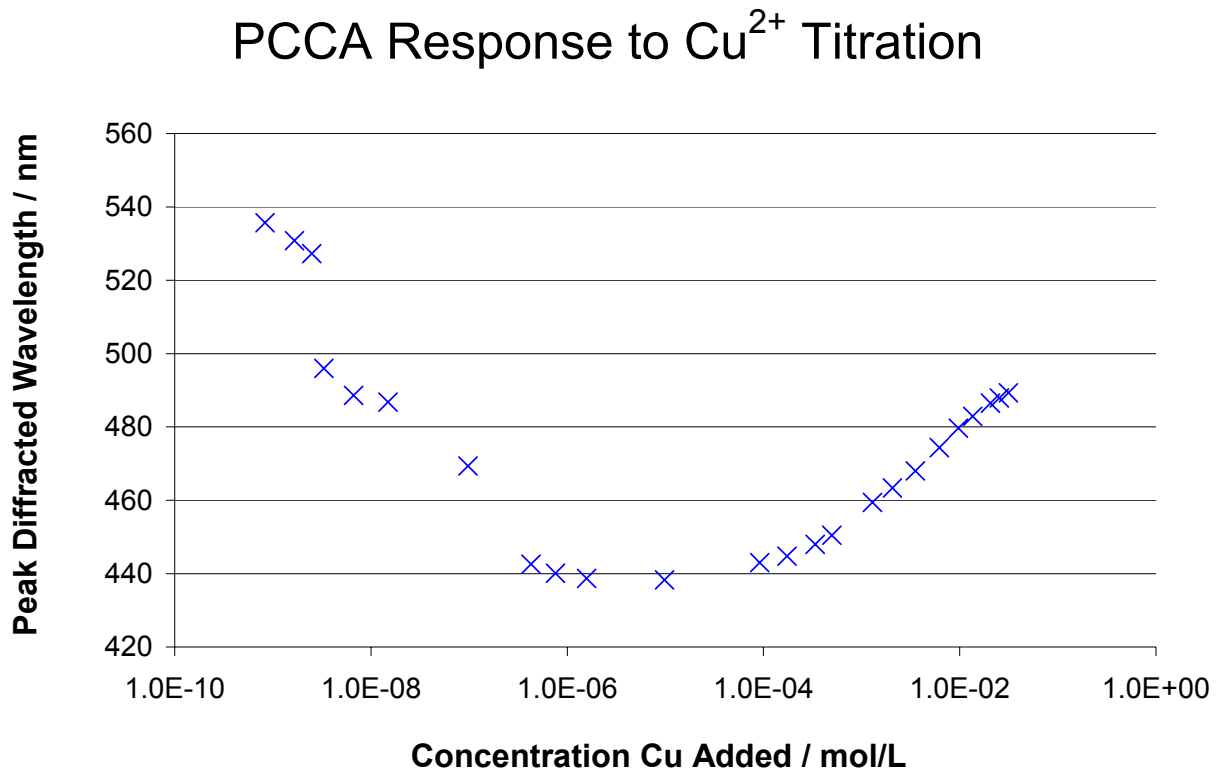

Figure 7.1. Response of a PCCA sensor for metal ions to low concentrations of Cu^{2+}. The sensor initially blue-shifts as additional hydrogel crosslinks are formed when two 8-hydroxyquinoline ligands bind each Cu^{2+} ion. The sensor acts as a dosimeter in this domain. At metal ion concentrations greater than ~50 µmol/L, the sensor red-shifts with increasing Cu^{2+} concentration as crosslinks are broken when Cu^{2+} ions are bound by a single 8-hydroxyquinoline. The sensor response in this range is reversible. An identical response is expected for Ni^{2+}.

We currently use the PCCA sensors in the domain where increasing metal ion concentration causes a red-shift in the peak diffracted wavelength. In this range, increasing the density of metal chelating groups on the hydrogel and decreasing the bisacrylamide crosslink density should increase the range diffracted wavelengths observed in this reversible response domain. Using chelating agents with a higher association constant should increase the slope of the sensor response in the relevant analytical range of metal ion concentrations (10^{-5} - 10^{-3} mol/L).

7.2.2 Testing of PCCA metal sensors with ischemic plasma samples

While the PCCA metal ion sensors may be optimized for larger shifts in diffracted wavelength over the relevant (0.1-1.0 mmol/L) analytical range, their response is sufficient to begin testing clinical plasma samples. We have submitted a proposal to the institutional review board at the University of Pittsburgh to collect and study blood samples from patients who are undergoing a clinically indicated cardiac stress test with myocardial perfusion imaging. Many of the patients undergoing such a test are expected to experience cardiac ischemia during exercise, and this ischemia will be determined by imaging with a radioactive contrast agent. We have proposed to collect two blood samples from patients who are undergoing this test in the course of their routine medical care. One sample will be collected before the test begins, and the second will be collected 15-20 minutes after maximum exertion. This will allow us to study plasma samples from subjects in the presence and absence of myocardial ischemia. While not all subjects will experience myocardial ischemia during the stress test, the results of the myocardial perfusion imaging will indicate which subjects experienced significant ischemia.

We will use the sensors as described in Chapter 5 to determine the change in the metal binding affinity of plasma from these subjects in the presence of ischemia. We will use two pieces of the same PCCA sensor to test the two plasma samples from each subject. This will ensure that the exact same sensor composition is present for each pair of plasma samples. After testing the samples, we will review the results of the myocardial perfusion tests to determine whether decreased myocardial perfusion correlates with a decrease in the metal binding affinity of plasma as determined by our sensors. We initially plan to study blood samples from about 50 subjects, 25% of whom are expected to show signs of myocardial ischemia during the stress test. This pilot study will aid in planning future clinical studies.

Finally, a large clinical study using the optimized sensor would be needed to confirm the utility of these PCCA sensors for rapidly detecting myocardial ischemia. This study should be performed in a clinical setting similar to where the sensors would potentially be used. This would likely be in an emergency medicine department. Other tests for myocardial ischemia, such as the cobalt binding assay, or tests for myocardial infarction, such as immunoassays of cardiac troponins, would be done simultaneously. This would help to determine the ability of the PCCA sensors to detect myocardial ischemia and to predict myocardial infarction in comparison with current tests.

7.3 CONCLUSION

The work described in the first six chapters contributes significantly to the development of point-of-care sensors for the management of diabetes and the rapid detection of myocardial ischemia. The future studies outlined above will complete the development of these sensors, and move them towards practical use in the diagnosis and management of two of the deadliest diseases we currently face. Non-invasive tear glucose sensors have the potential to improve glycemic control in patients with diabetes, and delay or prevent the complications of this disease. Sensors for the rapid detection of myocardial ischemia have the potential to speed diagnosis of this condition. The work presented here will enable rapid treatment if ischemia is detected and conservation of expensive medical resources if it is ruled out.

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