analytical chemistry

TECHNICAL NOTE

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Measurement of Tear Glucose Levels with Amperometric Glucose Biosensor/Capillary Tube Configuration

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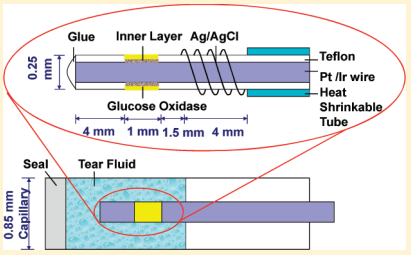
Supporting Information

ABSTRACT: An amperometric needle-type electrochemical glucose sensor intended for tear glucose measurements is described and employed in conjunction with a 0.84 mm i.d. capillary tube to collect microliter volumes of tear fluid. The sensor is based on immobilizing glucose oxidase on a 0.25 mm o.d. platinum/iridium (Pt/Ir) wire and anodically detecting the liberated hydrogen peroxide from the enzymatic reaction. Inner layers of Nafion and an electropolymerized film of 1,3-diaminobenzene/resorcinol greatly enhance the selectivity for glucose over potential interferences in tear fluid, including ascorbic acid and uric acid. Further, the new sensor is optimized to achieve very low detection limits of 1.5 ± 0.4 μM of glucose (S/N = 3) that is required to monitor glucose levels in tear fluid with a glucose sensitivity of 0.032 ± 0.02 nA/μM (n = 6). Only 4−5 μL of tear fluid in the capillary tube is required when the needle sensor is inserted into the capillary. The glucose sensor was employed to measure tear glucose levels in anesthetized rabbits over an 8 h period while also measuring the blood glucose values. A strong correlation between tear and blood glucose levels was found, suggesting that measurement of tear glucose is a potential noninvasive substitute for blood glucose measurements, and the new sensor configuration could aid in conducting further research in this direction.



Glucose monitoring technologies have drawn significant attention over the past several decades to help in the management of diabetes, which afflicts about 5% of the world's population.[1] Tight glycemic control is critical to the care of patients with diabetes, especially to prevent complications such as cardiovascular disease.[2] It is recommended that blood glucose levels be measured several times a day, which usually requires finger pricking coupled with measurement using a strip-test type glucometer (with either optical or electrochemical readout). However, in practice, patients may not follow these recommendations, and this might be largely due to the accumulated pain from the repeated finger pricks and blood collection.

A number of studies have been carried out to find a less invasive means to monitor blood glucose levels, including the use of infrared spectroscopy,[3,4] a GlucoWatch design that is based on electro-osmotic flow of subcutaneous fluid to the surface of the skin and detection of glucose with the enzyme−electrode system,[5] and measurement of tissue metabolic heat conformation,[6] but none of these techniques have yielded the quality of analytical results required to become a full substitute for blood glucose measurements.[7] Other investigations have suggested testing glucose in tear fluid as a substitute for blood, and this concept dates back to the 1950s.[8] This approach provides a unique possibility of developing a relatively simple noninvasive method of detecting glucose, if it can be clearly shown that tear glucose levels correlate closely with blood glucose values. If a good correlation between the two types of samples can be established, measurement of tear glucose levels could provide an attractive indirect measurement method for blood glucose levels within the normal as well as hyperglycemic and hypoglycemic ranges. For such a method to be effective, tear fluid needs to be collected using a nonstimulating method[9] so that increases in tear production do not further dilute out the naturally present glucose. At the same time, it is important to sample the tear fluid without inflicting any damage to blood capillaries within the eye, which might result in tear samples with much higher levels of glucose than actually present in the neat tear fluid sample (see below). With proper training, this should not be a major problem. Indeed, patients with type 1 diabetes who have to prick their finger a number of times per day would be highly motivated to learn and use such an approach if tear glucose values truly represent a measure of blood glucose concentrations.

Research has been conducted by a number of groups to develop detection methods for measuring the levels of glucose in tears. The requirements of tear glucose detection include a low detection limit (i.e., micromolar range), high selectivity over interferences such as ascorbic acid and uric acid, and the ability to measure small sample volumes as tear fluid can only be collected via a few microliters at a time. Published methods include capillary electrophoresis (CE) coupled with laser-induced fluorescence (LIF),[10] fluorescence sensors,[11] liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (ESI-MS),[12] holographic glucose sensors,[13] a miniaturized flexible thick-film flow-cell detector,[14] and a strip-type flexible biosensor.[15] Badugu et al. also reviewed the feasibility of using disposable contact lenses to monitor glucose through ophthalmic

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detection.[16,17] They suggested that this new approach can be considered as a significant alternative to diabetes care and management because many diabetics require vision correction and already wear contact lenses.

With the use of an enzymatic method, it was found in the 1980s that tear glucose levels were significantly higher in diabetic patients with higher blood glucose levels than normal patients.[18] However, levels of glucose in tears have been found to be typically 30−50 times lower than in blood. Baca et al. recently reviewed studies of the correlation between blood and tear glucose levels using different detection methods[9] and concluded that there is evidence of a correlation between average tear and blood glucose concentrations, but further characterization and justification is needed from animal and human studies to determine the potential utility of tear glucose measurements to help achieve glycemic control. In a recent paper, the correlation between the tear glucose concentrations and the average blood glucose concentrations was found to be stronger for noncontact lens wearers than for participants wearing contact lenses by using an LC−MS glucose detection method.[12] However, previous studies of critically ill patients using a high-performance liquid chromatography method with pulse amperometric detection (HPLC-PAD) to monitor tear glucose showed no significant correlation between tear and blood glucose concentrations.[19] As a result, further research is needed to evaluate whether measuring tear glucose concentrations can be considered a reasonable substitute for blood glucose monitoring.

Herein, we describe a relatively simple needle-type amperometric enzyme electrode for glucose that is capable of measuring the levels of glucose in tear fluid down to 1.5 μM, within a capillary tube containing ~5 μL of tear fluid. The sensor is utilized to assess the correlation between tear glucose levels and blood glucose concentrations in anesthetized rabbits. It will be shown that measurements with the electrochemical device suggest reasonably good correlation between the two types of samples within a given animal at the higher levels of glucose observed in these animal experiments; however, the ratio between tear glucose and blood glucose is found to vary considerably from animal to animal.

EXPERIMENTAL SECTION

Materials. Glucose oxidase (type VII, from *Aspergillus niger*), d-(+)-glucose, glutaraldehyde, bovine serum albumin (BSA), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), iron(III) chloride ($FeCl_3$), 37% hydrochloric acid (HCl), L-ascorbic acid, uric acid, Nafion (5 wt % solution in a lower aliphatic alcohols/H_2O mix), 1,3-diaminobenzene, and resorcinol were all purchased from Sigma-Aldrich (St. Louis, MO). Platinum/iridium (Pt/Ir) and silver (Ag) wires were products of A-M Systems (Sequim, WA).

Fabrication of Tear Glucose Sensor. The design of the tear glucose biosensor (see Figure 1) was based on previous configurations used to prepare electrochemical sensors suitable for subcutaneous measurements of glucose.[20,21] Briefly, a 10 cm long Teflon-coated Pt/Ir wire of 0.2 mm outer diameter was cut and a 1 mm cavity was created (by stripping the Teflon) at 4 mm from one tip. Starting 1.5 mm above the opening, a 15 cm, 0.1 mm o.d. silver/silver chloride (Ag/AgCl) wire was tightly wrapped around the sensor covering a length of 4 mm. The Ag/AgCl wire was prepared by dipping the Ag wire into a 1 M $FeCl_3$ in 0.1 M HCl solution. The straight section above the wrapped Ag/AgCl wire was then covered with a 5 cm long, 0.4 mm o.d. heat shrink polyester tubing (Advanced Polymers, Salem, NH).

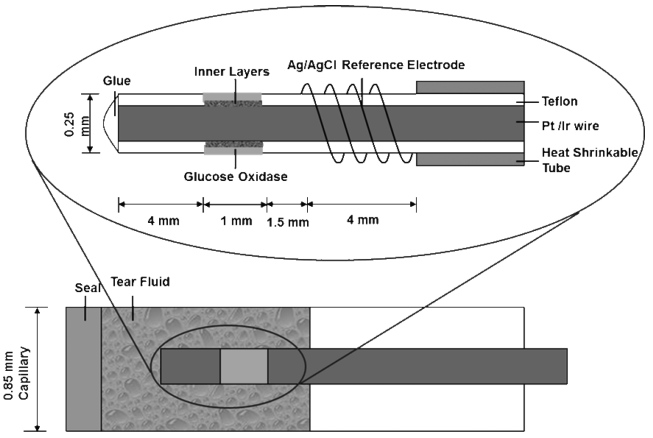

Figure 1. Configuration of tear glucose sensor/capillary. The glucose sensor design was adapted from that described in ref 20, except that no outer polymeric coating was used to decrease the glucose flux to the enzyme layer.

Inner polymeric layers deposited on the Pt electrode were used to eliminate interferences from ascorbic acid and uric acid. First, the cavity was coated with a thin layer of Nafion (~5 μm thick). Then, electropolymerization of a solution containing 1.5 mM 1,3-diaminobenzene and a similar concentration of resorcinol in PBS buffer (0.1 M, pH 7.4) was initiated using a voltammograph potentiostat (Bioanalytical Systems Inc., West Lafayette, IN) with a cycling voltage of 0 to +830 mV at a scan rate of 2 mV/s for 18 h.[22] The enzyme layer was created by first dropping 1 μL of a 3 wt % glucose oxidase solution containing also 3 wt % BSA in the cavity along the wire and drying this layer for 30 min. Then, the enzyme was cross-linked by adding 1 μL of 2% (v/v) glutaraldehyde solution and cured in air for 1 h. The sensor was then rinsed with deionized water and stored in 0.1 M PBS (pH 7.4) buffer for future use.

Calibration of Tear Glucose Sensor. The amperometric tear glucose sensors were calibrated using a four-channel BioStat potentiostat (ESA Biosciences Inc., Chelmsford, MA). The sensors were first polarized at a potential of +600 mV vs Ag/AgCl reference in a vial containing 10 mL of PBS buffer solution. Volumes of 5 μL of glucose standard solutions (5−800 μM) prepared in PBS were collected by individual 0.85 mm i.d. glass capillaries (World Precision Instruments, Sarasota, FL) and sealed with Critoseal (McCormick Scientific, Richmond, IL). The sensor was then taken out of the PBS, blotted briefly with Kimwipes (Kimberly-Clark, GA) to remove excess solution, and inserted into the capillary so that the solution completely covered the sensing region containing the immobilized enzyme (see Figure 1). After the sensor current was recorded 2 min after sensor introduction to the capillary sample, the sensor was rinsed with water three times and then put back into the stock PBS buffer to reach the steady-state baseline value in preparation for the next measurement within the capillary tubes. To test the sensor selectivity over interferences, standard solutions containing potential interfering species at their maximum possible levels in tear fluid[23,24] (i.e., 100 μM of uric acid, 100 μM of ascorbic acid, and 10 μM of acetaminophen (based on the dilution factor blood ratio)) were collected in capillaries, and the response current for

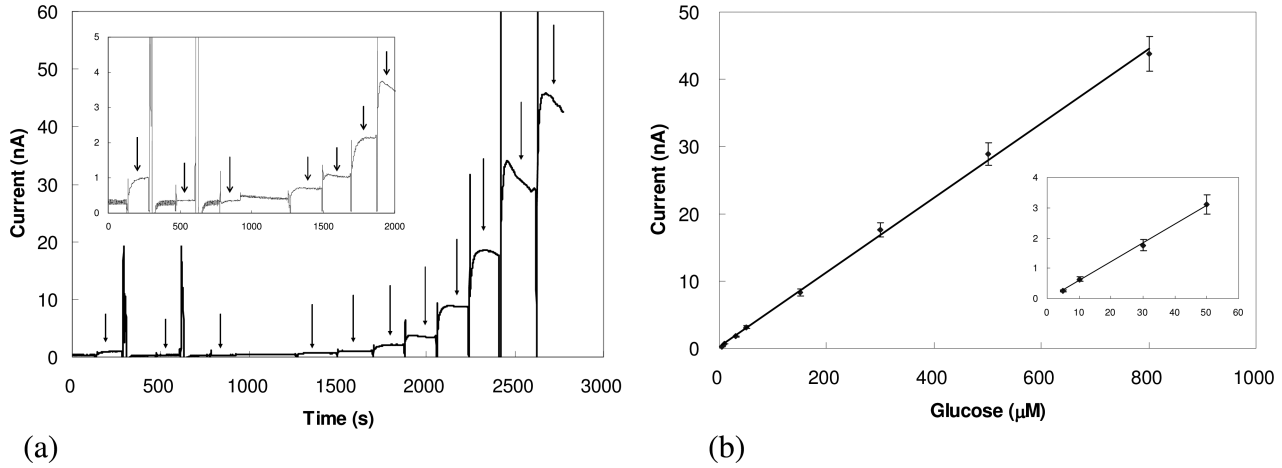

Figure 2. Calibration of tear glucose sensor using 5 μL of solution in capillary. (a) Solutions in the order of 100 μM uric acid, 100 μM ascorbic acid, 10 μM acetaminophen, and 5 μM, 10 μM, 30 μM, 50 μM, 150 μM, 300 μM, 500 μM, and 800 μM glucose. Inset: Interferences and low-end (5−50 μM) glucose response (note, the sensor is rinsed with water and soaked briefly in PBS buffer between each placement in the capillary tube containing the test interference or standard.) (b) Resulting calibration curve of tear glucose sensor. Inset: Low-end glucose calibration (5−50 μM). Error bars represent standard deviation (SD) of n = 3 replicate measurements of each standard within capillary tubes.

each interfering species was measured. On the basis of the sensitivity of the sensor to glucose and the amperometric signal observed for these interfering species, the % error that would occur for samples containing these levels of interferences and 100 μM tear glucose were calculated. To test the repeatability of such tear glucose sensors, the device was inserted into five separate capillaries containing 5 μL of 100 μM glucose and also five capillaries containing 20 μM glucose, with washing and baseline stabilization in PBS buffer in between the multiple measurements. The average reported glucose concentration was determined from a prior calibration curve made in capillary tubes using 5−800 μM glucose standards. It should be noted that once the test standard solutions (or tear fluid) are introduced by capillary forces into the glass capillary tubes, the distal end of the tubes are sealed by pressing the ends into a small volume of wax sealant (Critoseal). This allows the electrochemical sensor to be inserted from the top without forcing solution out from the distal end. Also, the volume of test solution does not have to be precisely controlled in this procedure, since the current at the 2 min time point after sensor insertion into solution is used for calibration or reporting test results.

Protocol to Assess Correlation between Tear and Blood Glucose Concentrations in Rabbits. A total of 12 white rabbits (Myrtle's Rabbitry, Thompson's Station, TN) were used in this study to test the correlation between tear glucose measured with the needle-type sensor and blood glucose measured with a commercial whole blood analyzer (see below). An anesthesia protocol as described elsewhere in detail[25] was followed for the experiments with the exception that the maintenance fluid rate was adjusted to 3.3 mL/kg/min. In short, the animals were initially anesthetized via intramuscular injections of 5 mg/kg of xylazine and 30 mg/kg of ketamine hydrochloride. Anesthesia was maintained by administering a diluted intravenous infusion of ketamine hydrochloride (2 mg/mL) at a rate of 1.53 mg/kg/h. The paralytic, pancuronium bromide 0.33 mg/kg, IV), was administered to have the animal totally dependent upon mechanical ventilation, which was done via a tracheotomy and using a Sechrist Infant Ventilator, model IV-100. All rabbits were under anesthesia for 8 h. The tear glucose sensor was polarized at +600 mV in PBS buffer through the duration of the entire experiment. The sensor was calibrated in capillary tubes with 100 μM glucose in the middle of the 8 h experiment. Every 30 min, 0.6 mL of blood was drawn and the blood glucose level was measured using an Radiometer ABL 725 blood analyzer (Radiometer America Inc., Westlake, OH) that employs a macro-electrochemical enzyme electrode to quantitate blood glucose. At the same time, 5 μL of rabbit tear fluid was collected in the capillary and the current from the glucose in the tear fluid was recorded using the tear glucose sensor. The tear glucose level was calculated from the one point calibration result. Statistical data analysis was carried out to examine the correlation between the blood and tear glucose values within a given animal and across all 12 animals involved in the study.

RESULTS AND DISCUSSION

Analytical Performance of Tear Glucose Biosensor. The typical calibration curve for the tear glucose biosensor in capillary tubes is shown in Figure 2. The detection limit is 1.5 ± 0.4 μM of glucose (S/N = 3) (see inset in Figure 2 for low-level calibration). It should be noted that this low detection limit is achieved by not coating the outer surface of the sensor with an additional membrane that restricts diffusion of glucose to the enzymatic layer. Such an additional coating is required for blood and subcutaneous glucose sensing in order to ensure that oxygen is always present in excess compared to glucose in the enzymatic layer to achieve a linear response to high glucose concentrations. However, given the much lower levels of glucose in tear fluid, no outer membrane is needed to retard glucose diffusion, since oxygen levels will be always in excess in such samples. This ultimately enables the very low detection limit of the sensor. The glucose sensor design employed in this work has an average sensitivity of 0.032 ± 0.02 nA/μM of glucose (n = 6). It should be noted that greater sensitivity (by nearly factor of 5) and even lower detection limits (0.62 μM) than the 1.5 μM value indicated above can be achieved for the same sensor design if 5 wt % glucose oxidase, rather than 3 wt %, is used in the 1 μL of enzyme

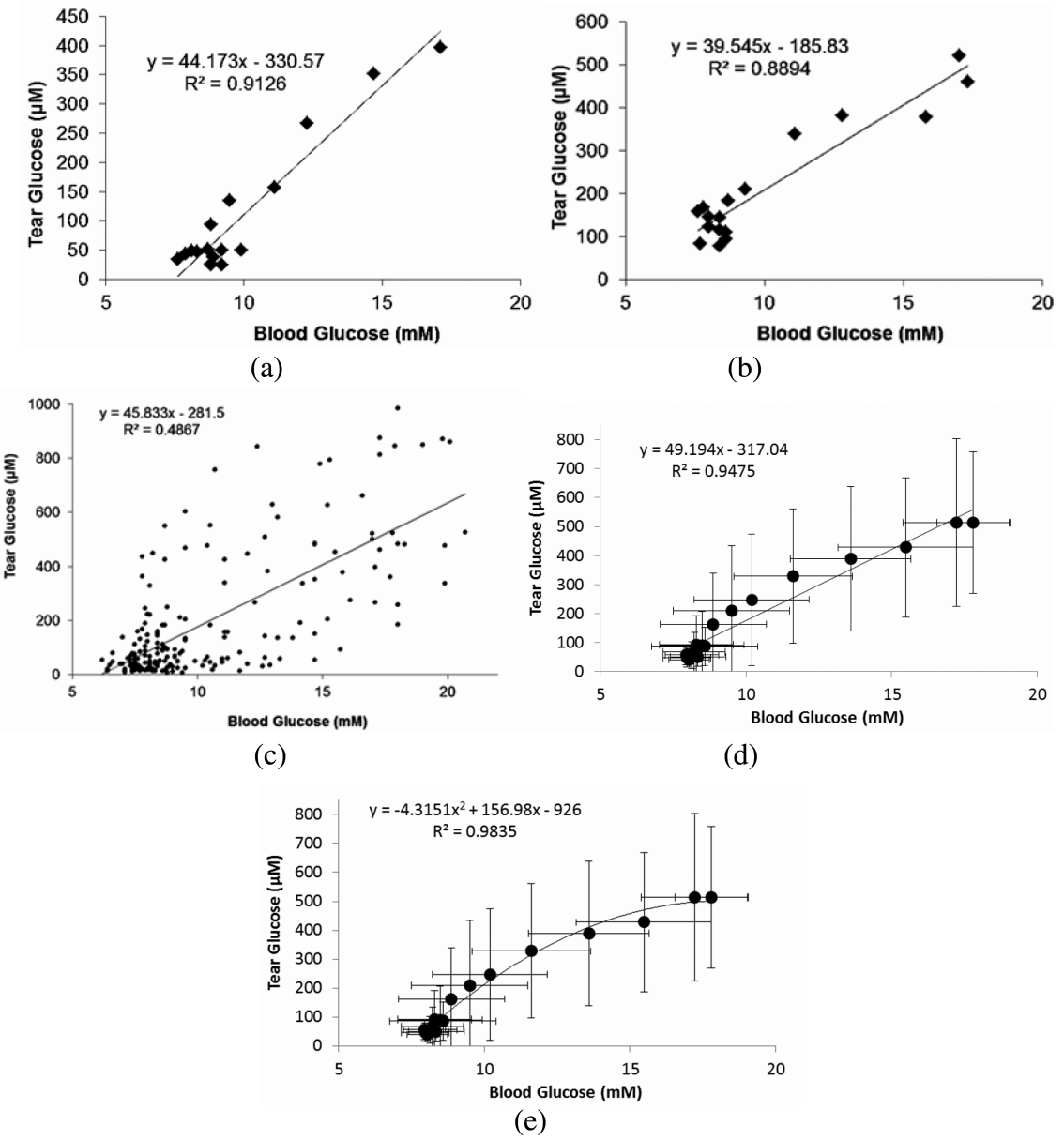

Figure 3. Correlation between tear and blood glucose levels using a rabbit model. (a and b) Results from two individual rabbit experiments. (c) All the data points of tear and blood glucose values of the total 12 rabbits. (d) The average values of both tear and blood glucose levels for all animals in study at every half hour time point fitted to least-squares linear regression. (e) A 2nd order polynomial correlation between average tear and blood glucose levels for the same data shown in part d.

layer employed in fabricating the sensor (see Figure 1s in the Supporting Information). The linear range for either sensor formulation can reach to at least 800 μM, which is nearly 7-fold greater than the average normal value of 138 μM found previously for tear glucose levels in humans.[10] From the repeatability test of the tear glucose sensors, the sensors exhibited an acceptable repeatability with an average of 102.5 $\pm$ 3.2 μM measured for the 5 measurements in individual capillaries containing $\sim$5 μL of 100 μM glucose solution each. Similar reproducibility was observed for 5 measurements of a lower concentration glucose standard prepared at 20 μM within capillaries, with an average value of 19.10 $\pm$ 0.23 μM.

Any glucose sensor designed for measurements in physiological tear fluid must exhibit acceptable selectivity over existing electroactive species typically present in tears. At the potential of +600 mV vs Ag/AgCl reference electrode, those interferences might also be oxidized at the working electrode used to detect the hydrogen peroxide generated from glucose oxidase reaction with glucose, adding error to the output current. It has been reported in the literature that ascorbic and uric acid concentrations in tear fluid are $\sim$20 and 70 μM, respectively.[23,24] As a result, 100 μM of both ascorbic acid and uric acid were used to test the selectivity of a tear glucose sensor. For small neutral molecule interferences, 10 μM of acetaminophen was employed for testing, assuming that this species would be present in tear fluid at levels similar to the relative dilution ratio of blood glucose levels. The error percentage was calculated by dividing the current of certain interferences by that observed for a 100 μM standard of glucose.

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The presence of the Nafion and electropolymerized 1,3-diaminobenzene/resorcinol inner layer enabled the sensor to exhibit excellent exclusion of interferences with the % errors for ascorbic acid, uric acid, and acetaminophen of 7.56, 11.16 and 4.85%, respectively. These results indicate that the tear glucose biosensor has acceptable selectivity over major electroactive interferences found in tear fluid and that results obtained for tear samples will likely reflect the true level of glucose present in such samples.

Correlation of Tear Glucose and Blood Glucose from the Rabbit Model. Figure 3a,b shows the Pearson's correlation between tear and blood glucose from two individual rabbit experiments. The determined r^2 values are 0.9126 and 0.8894, respectively ($p \ll 0.05$), indicating significant correlation between tear and blood glucose concentrations. Both examples show excellent fitting to the linear regression model. Figure 3c shows all the blood−tear glucose values from the 12 rabbit experiments. There seems to be a low correlation between blood and tear glucose concentrations when the data from all animals tested are combined, based on the results obtained using Pearson's correlation analysis ($r^2 = 0.4867$, $p \ll 0.05$). Furthermore, it is difficult to establish a simple mathematic function model, such as a linear relationship, between the tear and blood values for the entire data set. This is due to the fact that there was a significant difference in the correlations for individual rabbits. This implies that even though the tear and blood glucose levels in each rabbit demonstrate a reasonable linearity in correlation, the variation among individual animals undermines this general trend as a whole and this resulted in a low global tear−blood glucose correlation.

It should be noted that there is a common trend of blood and tear glucose concentration decay from the beginning of the 8 h experiment for all the rabbits tested, with rather high levels observed throughout the test period. It is known that anesthesia will elevate glucose levels,[26] and this likely causes the elevated blood glucose levels that were observed during the initial test period, with a continuous decrease to still slightly elevated levels (7−8 mM) at the end of the 8 h test period. Unfortunately, the approved animal protocol employed in this work did not allow administration of insulin which would help reduce glucose concentrations to normal or below normal levels. Further, the slow decrease in glucose concentration with time and the 30 min sampling period employed in these animal experiments did not enable the measurement of the expected lag time for any sudden change in blood glucose levels and the corresponding change in tear values. However, it is anticipated that lag times for changes in tear glucose would likely be similar to the time delays observed for subcutaneous measurements of glucose using implantable electrochemical glucose sensors.[9]

As a result of the continuous decrease in blood glucose levels in the rabbits under anesthesia, average values of both blood and tear glucose values were taken at each half-hour time point. The shared trend of glucose decay in both blood and tear glucose values indicates that the blood and tear glucose levels increase or decrease in tandem. Figure 3d shows the average of blood−tear glucose levels at 30 min increments. A Pearson's correlation analysis reveals a significant relationship between tear and blood glucose concentrations ($r^2 = 0.9475$, $p \ll 0.05$) and a linear regression shows excellent fitting. With the use of a second order polynomial correlation, the fitting model between tear and blood glucose levels is even better ($r^2 = 0.9835$) (Figure 3e). Although this fitting shows a slightly higher correlation coefficient, it makes the model one order more complex, with only slight gains. As a result, in future applications, the linear model can still be used with acceptable accuracy.

In the potential real-world application of the biosensor method for monitoring glucose levels of diabetic patients, after the correlation between tear and blood glucose levels for each individual is established (presuming, like rabbits, the exact correlation and dilution factor from patient to patient may vary), an abnormal tear glucose concentration range can be set up to detect dangerous blood glucose levels from the correlation. Thus, tear glucose levels can be measured multiple times per day to monitor blood glucose changes without the potential pain from the repeated invasive blood drawing method. Indeed, blood glucose levels can still be measured using the traditional blood collection method in order to trigger proper therapy when tear glucose detection suggests that blood glucose levels are out of the normal range.

CONCLUSIONS

A simple electrochemical tear glucose biosensor coupled with a tear fluid collection capillary configuration has been used to noninvasively monitor glucose levels in tears from rabbits. The needle-type amperometric sensor exhibits excellent selectivity over known electroactive interferences, a low detection limit, a wide dynamic range, excellent repeatability, and at present requires a 4−5 μL sample volume. With further miniaturization of the sensor diameter, it is likely that measurements in as little as 1−2 μL of fluid should be possible, a volume more suitable for routine tear glucose measurements in humans, and this should provide a convenient measurement device to aid researchers interested in further investigating the clinical utility of tear glucose measurements as an alternate to blood measurements. In this work, the correlation between tear and blood glucose levels at the higher end range (owing to continuous use of anesthesia in the animal model employed) has been established in a rabbit model, and data analysis suggests that a significant correlation between tear and blood glucose levels does exist but that the exact correlation varies from animal to animal. Hence, the use of tears as an alternate sample to assess blood glucose in human subjects will likely require that the ratio of glucose in tears and blood be established first for a given individual, so that the appropriate algorithm can be employed to report values that more closely reflect the true blood levels present. Near term animal studies will focus on establishing whether similar correlations exist in the rabbit model at more normal and even hypoglycemic blood glucose levels, via use of insulin in our existing rabbit model animal experiments. Additional research to employ a coulometric measurement method with a variant design of the glucose sensor that can be placed within capillaries containing 1 μL of tear fluid is envisioned. Such a coulometric approach may not require any sensor precalibration, provided that a reproducible and constant volume of tear fluid can be sampled.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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