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Continuous glucose sensor using novel genetically engineered binding polypeptides towards *in vivo* applications

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ABSTRACT

As the importance of blood-glucose control for both diabetic and non-diabetic patients continues to increase, there is a need for more advanced glucose-sensing technologies. In particular, an *in vivo* glucose sensor is needed that exhibits high accuracy when operating in a continuous manner for a relatively long period of time (3–5 days). Development of such sensors has been hampered, as low accuracy and sensor drift become major problems with *in vivo* environments, especially for enzyme-based electrochemical glucose sensors. This paper reports on the use of a novel, binding polypeptide-based, fluorescent, glucose-sensing system that promises to overcome many drawbacks of an enzyme-based system while showing the potential for high accuracy, especially at hypoglycemic levels.

Fluorescently labeled glucose recognition polypeptide elements were immobilized in a polyacrylamide hydrogel matrix placed on the tip of an optical fiber to realize a continuous glucose-sensing device towards *in vivo* applications. *In vitro* validation was performed in both buffered solutions and whole blood to characterize sensor parameters such as sensitivity and response time. Testing demonstrated that the reagentless polypeptide-based glucose-sensing system has extreme sensitivity in the hypoglycemic levels while providing high precision across the entire human physiologic glucose range. Additionally, the sensor was shown to function at physiologic temperature (*viz.*, 37 °C) and displayed high selectivity for glucose without interference from other sugars (*viz.*, fructose).

This represents the first report of implementing immobilized glucose binding protein-like elements in a sensing device for continuous glucose monitoring, and establishes proof-of-concept as an excellent alternative to overcoming problems of current long-term, continuous glucose-sensing technologies.

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1. Introduction

Much biosensor research in industry and academia has focused on the development of robust glucose sensors, with a resulting abundance of reported technologies and medical applications [1–5]. The currently available and successful point-of-care (POC) handheld glucometers are electrochemical-based and commonly rely on either glucose oxidase (GOX) or glucose dehydrogenase (GDH) enzymes. However, these devices have significant drawbacks. In practice, their use is burdensome and often not accurate or specific, especially in hospital environments, as they result in inconsistent readings with errors that can exceed 20% [6,7]. These sensors also have severe limitations in differentiating between low-normal glucose levels and hypoglycemia, and some suffer specificity issues as highlighted by the FDA's warning regarding POC meters [8]. In particular, a possible interfering sugar is fructose, and can fluctuate in high concentrations depending on, for example, a patient's diet, which may consist of high amounts of fruit juices.

The main motivator of glucose sensor research and development is the world's large and growing diabetic population, with an estimated 350 million people worldwide suffering from diabetes mellitus by 2025 [9,10]. In addition, studies have indicated the need for blood-glucose control of all in-hospital patients (including non-diabetics), especially those in critical conditions [11–16]. By controlling blood-glucose levels within a narrow range, a concept known as tight glycemic control (TGC), studies have demonstrated decreases in morbidity, mortality, time of hospital stay, and other complications for patients in critical care conditions [11–15]. Some recent studies have suggested that attempting TGC is difficult and is

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associated with increased risks of hypoglycemia and related complications [6,17–19]; by reading these reports in detail, one realizes this is in large part due to the lack of an accurate and reliable continuous glucose-sensing technology [6,20,21]. In the majority of these "negative" studies, clinicians attempted TGC with the same inadequate handheld POC glucometers discussed above, with measurements taken infrequently, such as only once per hour [17]; the infrequent measurements and large sensing errors make TGC difficult. Despite disagreements of how tightly blood glucose should be controlled or within which range [17,18], it has been generally accepted that blood-glucose control is important, and that the benefits of TGC can only be realized if such efforts do not increase the risk of hypoglycemia. This further underscores the need for accurate and reliable continuous glucose monitoring (CGM).

There are arguably no CGM devices on the market capable of maintaining the required accuracy over time. Of note, however, are Dexcom's SEVEN+ and Medtronic's MiniMed Guardian CGM systems, which measure subcutaneous glucose electrochemically in interstitial fluid. Substantial sensor drift and poor reliability have hindered FDA approval as stand-alone glucose measurement devices, as they are approved only for adjunctive use [22]. Thus, there remains a need for better, more advanced glucose-sensing technologies that can meet today's continuous *in vivo* applications and healthcare requirements.

There are many alternative technologies under development that may overcome problems of the common electrochemical-based enzyme glucose-sensing systems discussed above. Some of these sensing methods include near infrared (NIR) spectroscopy, optical coherence tomography (OCT), and fluorescence-based systems [23]. Specific examples of fluorescence methods include fluorescent boronic acid-based sensors, and fluorescently labeled binding proteins and elements, the later of which are the focus of the work presented here [24–26]. Other common fluorescence-based glucose-sensing approaches involve the use of Concanavalin A (ConA). Indeed, in the field of fiber-optic based glucose sensors much progress has been made by adopting ConA in various forms onto fiber-optic sensors [27–30]. More extensive overviews of various glucose-sensing technologies can be found in the literature [3–5,23,31,32].

1.1. Glucose binding polypeptides

This paper focuses on the use of rationally engineered glucose binding protein (GBP)-like sensing polypeptide elements towards invasive, *in vivo* CGM devices and applications. Wild-type GBPs are found in the periplasmic space of many gram-negative bacteria, where they are responsible for mediating glucose uptake. GBPs bind in a very specific manner to D-glucose. GBP itself, approximately 33 kDa in size, has an approximate ellipsoidal shape, with two large, globular domains connected by three varying peptide segments that act as a flexible hinge, thus creating a cleft between the two large domains; the two domains remain separated or "open" in the absence of glucose. Upon glucose binding, a conformational change occurs whereupon the two larger domains "close" around the ligand (Fig. 1). The conformational change produced when GBP binds glucose can be transduced into a sensing signal by, for example, engineering the protein to feature an environmentally sensitive fluorophore near the hinge region where movement is greatest. Indeed, fluorescence-based GBP sensing molecules have been shown previously [33–36], such as the one by Daunert and co-workers [24].

Fluorescently labeled, glucose binding polypeptide-based biosensors can potentially overcome many of the disadvantages of traditional enzyme-based glucose-sensing systems: they are reagentless, do not consume glucose, and do not require chemical conversion. In contrast, enzyme-based sensors consume glucose

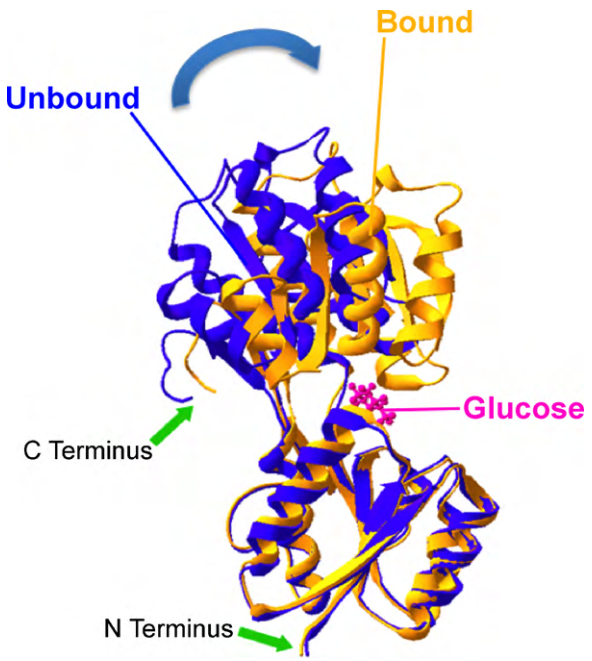

Fig. 1. Overlaid glucose binding protein crystal structures. Schematic shows both the glucose-bound and unbound states along with the glucose-binding pocket.

and other reagents (*e.g.*, O_2 or other electron mediators) that require a continuous supply. With GBP, only physical binding and unbinding of glucose takes place, representing benign entropic changes (ΔS) rather than enthalpic changes (ΔH), which, in enzyme systems, typically lead to degradation, inaccuracy, and sensor drift. The GBP system previously reported by Daunert and co-workers has a high specificity for glucose, relying on the molecular structure of the binding pocket, and the sensing mechanism is based on a strategically placed fluorescent label on a specific peptide site within the GBP amino acid sequence [24]. When a population of GBPs that have been strategically labeled with a fluorophore undergo conformational changes during glucose binding/unbinding, fluorescence intensity changes in proportion to glucose concentration, thus enabling glucose sensing. This transduction mechanism is the result of GBP binding around the glucose molecule as well as the strategically placed fluorophore, and in turn causing quenching of the fluorophore intensity.

In the field of GBPs, there has been work in engineering of polypeptide sequences to enhance performance and accept various signal transduction markers (*i.e.*, optical and electrochemical labels) [33–38]. There have also been some reports in the literature on the immobilization of GBPs towards a glucose-sensing system [39]. However, to the best of the authors' knowledge, there have been no reports to date on the successful immobilization of GBPs or GBP-like sensing elements within a fixed matrix such that a continuous glucose-sensing device is realized. Indeed, this paper reports on the use of a novel, genetically engineered, GBP-like sensing molecule (herein referred to as glucose recognition polypeptide, or GRP) immobilized within a hydrogel matrix and coupled to a fiber-optic measurement system for continuous glucose sensing towards *in vivo* applications [40]. The GRP-based glucose-sensing system demonstrates extremely high precision and sensitivity in the low glucose (hypoglycemic) levels, retains functionality *in vitro* in blood, has a fast response time (min), and is shown to be completely reversible and relatively stable. Finally, the GRP-based sensor functions at physiologic temperature (*viz.*, 37 °C) and shows no interference from fructose, a possible physiologically relevant interfering saccharide.

2. Materials and methods

2.1. Recognition polypeptide preparation and labeling

For this study, a novel GRP sensing molecule was prepared and fluorescently labeled using a similar protocol as previously reported by Daunert and co-workers [24]. The GRP used was rationally engineered to include sequence and structure changes to facilitate not only acceptance of a fluorophore, but to exhibit additional beneficial characteristics such as increased sensitivity within the clinically relevant glucose levels (approximately 2–20 mM glucose) and improved overall stability; the polypeptide engineering performed was based on the patent application by Daunert et al. [40].

Briefly, plasmids were constructed using PCR-based site-directed mutagenesis. *Escherichia coli* vectors were used to express the GRP, and after cell lysis via sonication, the GRPs were isolated, purified, and verified using a filtering system, solid-phase capture, and SDS-PAGE, respectively. The GRP sequence engineering included introduction of a unique cysteine (containing a thiol side chain) near the ligand binding pocket to facilitate a conjugation reaction for site-specific labeling with N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) (Invitrogen, CA, USA-prod #D-10253), a commercially available, environmentally sensitive, thiol-reactive fluorophore. After labeling with MDCC, the GRP-MDCC was isolated using a Sephadex G-25 column (Sephadex, IN, USA-prod #3117936001) and eluted into 10 mM HEPES (Sigma–Aldrich, MO, USA-prod #H3375) buffer containing 0.2 mM $CaCl_2$ (Sigma–Aldrich, MO, USA-prod # C1016) at pH 8.0. Additional GRP production details can be found in previous reports by Daunert et al. regarding the preparation and fluorescent labeling of binding protein elements [24,40,41].

In preparation for covalent incorporation into an acrylamide hydrogel, available lysines on the GRP-MDCC were conjugated with acrylic acid (AA) to create AA-GRP-MDCC. When present during hydrogel polymerization, acrylic acids are incorporated covalently into the hydrogel; thus, AA-GRP-MDCC can be covalently immobilized within the hydrogel matrix. To perform AA conjugation, 100 μL of GRP-MDCC in buffer (the same 10 mM HEPES containing 0.2 mM $CaCl_2$ at pH 8.0 from above) at a concentration of $\sim 3 \times 10^{-5}$ M was reacted with the NHS-ester of acrylic acid (Sigma–Aldrich, MO, USA-prod #A8060) in a 1:10 molar ratio by stirring in buffer for 1 h. Care was taken to ensure that hydrolysis of the NHS-ester of acrylic acid did not begin until after its addition to the GRP-MDCC. After 1 h of conjugation, the AA-GRP-MDCC was dialyzed against 1 L of the same HEPES buffer using dialysis units according to the manufacturer's protocol (Pierce Scientific, IL, USA-prod #69558); dialysis was performed for several hours with 3× buffer changes to remove any unreacted species. The AA-GRP-MDCC was then ready for incorporation into the hydrogel.

2.2. Fiber-optic hydrogel sensor preparation

In order to utilize GRP-MDCC as a continuous sensor, AA-GRP-MDCC was immobilized in an acrylamide hydrogel matrix attached to the tip of a fiber-optic cable. Polyacrylamide hydrogels have been shown to provide hydrophilic, protein-friendly environments that preserve protein/enzyme functionality [42] while providing a stable environment that tends to degrade minimally overtime in solution, a required characteristic for a long-term, continuous glucose sensor [43]. The AA-GRP-MDCC was covalently incorporated into the acrylamide hydrogel by mixing and polymerizing with the hydrogel precursors: acrylamide monomer (Sigma–Aldrich, MO, USA-prod #148660), bis-acrylamide crosslinker (N,N′-methylenebis-acrylamide) (Sigma–Aldrich, MO, USA-prod #M7279), and a UV-sensitive free-radical initiator (2,2-diethoxyacetophenone, or DEAP) (Sigma–Aldrich, MO, USA-prod #227102); additionally, glycerol was added to provide GRP stability and reduce evaporation during polymerization (Sigma–Aldrich, MO, USA-prod #G5516). All reagents were used as received by the manufacturer. HEPES buffer was used as the hydrogel solvent, with the following approximate hydrogel precursor solution component concentrations: 12 μM AA-GRP-MDCC, 4 M acrylamide, 0.1 M bis-acrylamide, 2% DEAP, and 2% glycerol.

Finally, a small aliquot (<1 μL) of the mixed hydrogel precursor solution was placed on the bare, polished end of a 1-mm diameter plastic optical fiber (POF) (Timbercon, OR, USA-custom), and exposed to UV light to drive polymerization. A UV lamp with nominal 365 nm radiation (VWR, PA, USA-prod #36585-815) was used for 10 min. During this polymerization step, the hydrogel becomes crosslinked, incorporating the acrylic acids of the AA-GRP-MDCC. After polymerization, the fiber-optic hydrogel sensors were conditioned in HEPES buffer containing D-glucose (Sigma–Aldrich, MO, USA-prod #G8270) overnight before testing. All sensors were stored in solution at all times, and refrigerated when not undergoing testing.

2.3. Measurement system apparatus

A fiber-optic measurement system was implemented to allow interrogation of the immobilized GRP fluorescence intensity at the fiber-optic tip. A bifurcated fiber-optic cable system was used in which one leg of the system provided excitation light via a Xenon flash lamp (Ocean Optics, FL, USA-prod #PX-2) filtered to 425 nm for MDCC excitation using a bandpass filter (Chroma, VT, USA-prod #D425/40×) placed in a filter holder (Ocean Optics, FL, USA-prod #FHS-UV). The second leg of the bifurcated fiber-optic cable system was attached to a USB-driven, CCD spectrophotometer (Ocean Optics, FL, USA-prod #USB4000) for detection of the fluorescence intensity. The common leg of the system was attached to the hydrogel GRP sensors (Fig. 2). With the exception of the bare-ended sensor fibers, all fiber-optic cables used were custom glass fibers from Ocean Optics. No filter was placed on the detector leg, as the spectral spread between the excitation and MDCC fluorescence peaks was wide enough to allow differentiated intensity measurements. Moreover, this allowed measurement of the excitation peak intensity (as reflected off of the sensor fiber tip), such that a ratiometric approach could be taken for intensity measurements to correct for excitation intensity fluctuations in the system.

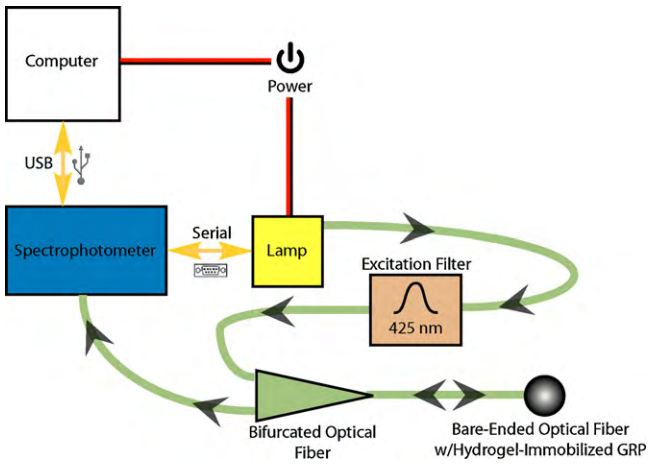

Fig. 2. Fiber-optic hardware system. Diagram shows the measurement system set-up used for fluorescence excitation and data collection of glucose-response behavior. Various fiber-optic sensors were tested by attachment to the permanent hardware system.

2.4. Testing, data acquisition, and processing

To perform testing, the entire measurement system apparatus was placed inside a dark holding box, with the hydrogel GRP sensors placed inside beakers of test liquids; care was taken to secure all fiber-optic cables to prevent movement during testing. In the case that buffered glucose solutions were used, D-glucose was added to HEPES buffer, and the hydrogel GRP sensor was placed inside a beaker containing 20 mL of the test solution. To vary the glucose levels, the sensors were simply placed in different bottles of varying glucose solutions; all buffer solutions were unstirred and kept a room temperature. For fructose testing, fructose (Sigma–Aldrich, MO, USA-prod #F0127) was added to the same buffered glucose solutions. In the case of blood testing, porcine blood (Lampire, CA, USA-prod #7204906) was heated at 37 °C and stirred for the duration of testing. Blood-glucose test solutions, also 20 mL in volume, were created by first mixing glucose in standard saline solution made by adding D-glucose to a 1% NaCl (Sigma–Aldrich, MO, USA-prod #S7653) solution at pH 7.4; the saline glucose solution was then added to spike the blood with glucose. While addition of the saline to the porcine blood lowered the hematocrit, care was taken to ensure each blood-glucose standard received the same volume of saline glucose solution, such that the relative hematocrit levels were equivalent. As with the buffered glucose solutions, varying blood-glucose levels were achieved by exposing the sensors to samples of different blood-glucose concentrations.

To acquire data, custom software was written with National Instruments' LabVIEW using a PC computer, with the detector and lamp controlled through the USB port. The entire spectrum was collected continuously with sampling every 10–60 s, with varying degrees of averaging, noise compensation, and spectral integration; intensity was measured for both the excitation peak (~425 nm) and the MDCC emission peak (~485 nm). Each data point consisted of a ratio of the emission intensity to the excitation intensity, such that fluctuations in the lamp intensity could be minimized; this simple method eliminated gross data anomalies, but did not eliminate fine detail intensity fluctuations. Specific details on each test performed can be found in Section 3.

After data collection, intensity measurements were processed and plotted using macros in spreadsheet software. While visual inspection of the glucose-response data indicated statistically relevant effects between the various glucose levels, additional statistical analysis was performed. For the glucose-response data, an analysis of variance *F*-test was performed, treating each glucose level as a sample; this provides insight as to whether the grouped glucose levels are statistically distinct. To gain further insight into the extent of distinction between the glucose levels, an additional *t*-test was performed. As the sensors reported here exhibited the least amount of sensitivity in the high glucose levels, a two-tailed, Student's *t*-test was performed on the two highest glucose levels, using the pooled-variance estimate. The same *t*-test was performed on the fructose data, with the two samples being glucose with and without fructose. In all cases, *P* values were then calculated using spreadsheet software macros.

3. Results

In this section, the details and results of *in vitro* testing in buffered glucose solutions are first presented, including tests to characterize glucose-sensing behavior, sensitivity, precision, and response time. Sensitivity to fructose, a common interfering saccharide, is also explored. Then, *in vitro* blood testing results are presented, showing the sensor's ability to function in complex solutions at higher physiologic temperatures.

Both the GRP-MDCC and AA-GRP-MDCC sensing elements retained their glucose-response activities in solution, verifying that sensing capabilities had not been lost (data not shown). Next, the AA-GRP-MDCC elements were immobilized in a polyacrylamide hydrogel matrix on the tip of a fiber-optic; Fig. 3 shows a resulting prototype sensor.

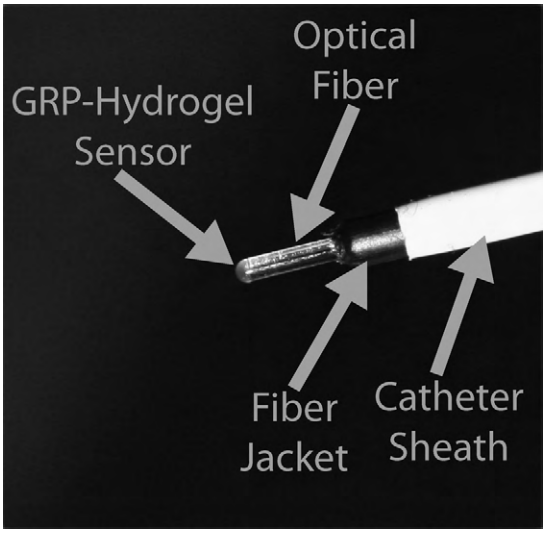

Fig. 3. Photo of a prototype GRP hydrogel fiber-optic sensor. The fiber-optic sensor is shown inserted into a diaphragm-sheath catheter with a close-up of the fiber-optic hydrogel sensing tip; fiber diameter is 1 mm.

To characterize the response and function of the GRP hydrogel sensors, an *in vitro* test was performed in buffered glucose solutions in which the sensor was exposed to high (20 mM, or equivalently 360 mg/dL) and low (2 mM, or equivalently 36 mg/dL) glucose levels, with the sensor allowed to stabilize in each solution for a total of 15 min while data were collected (Fig. 4). The main purpose of this test was to determine response time of the sensor, which was found to be 10 min between the high and low glucose values (*i.e.*, time for stabilization of the signal). As expected, the GRP sensor displayed a higher fluorescence intensity at the lower glucose level.

Next, a longer and more extensive test was performed in which the sensor was exposed to several different glucose levels. As in the experiment above, a fiber-optic GRP hydrogel sensor was tested *in vitro* in buffered glucose solutions of the following levels: 20 mM (360 mg/dL) → 15 mM (270 mg/dL) → 10 mM (180 mg/dL) → 5 mM (90 mg/dL) → 2 mM (36 mg/dL) and back through each glucose level

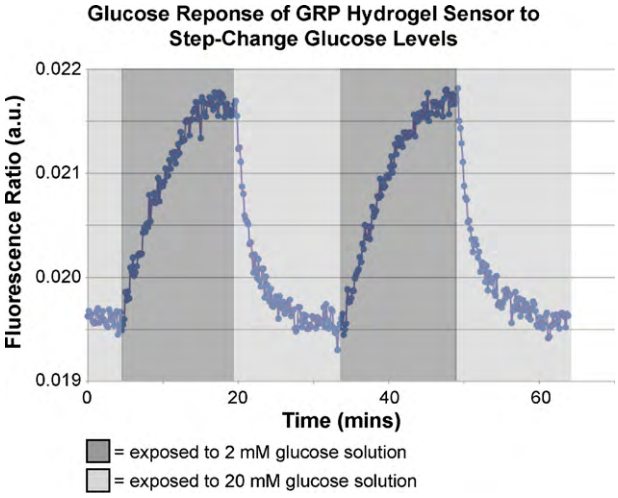

Fig. 4. Dynamic glucose-response data of a GRP hydrogel sensor. In this test, the sensor was exposed repeatedly to high (20 mM) and low (2 mM) buffered glucose solutions; data points were acquired every 10 s, and the entire test lasted 65 min.

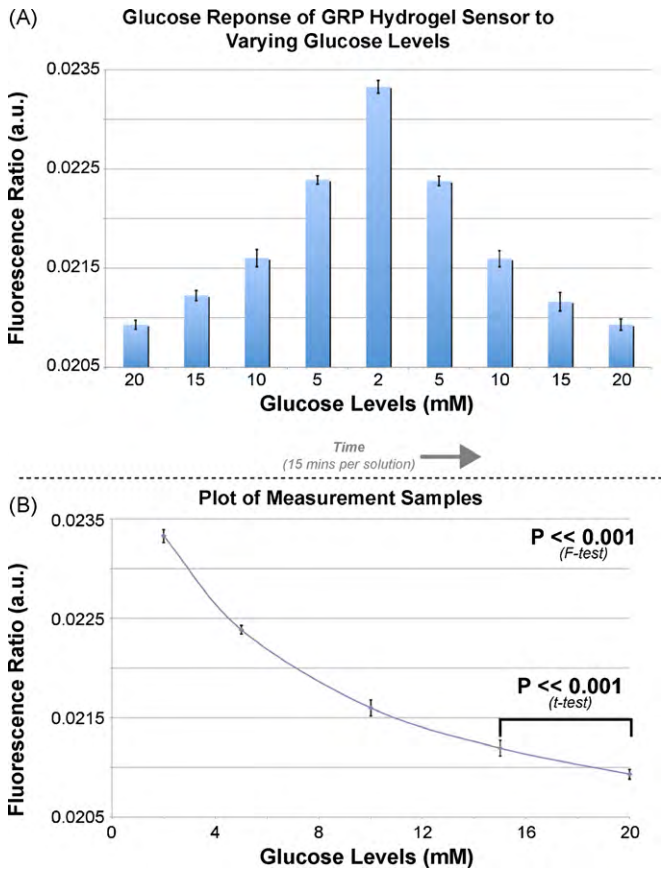

Fig. 5. Glucose-response data showing sensitivity of a GRP hydrogel sensor. The sensor was exposed to varying levels of buffered glucose solutions. The sensor was allowed to stabilize in each solution for 12 min and then data were taken for 3 min. (A) The data were averaged and plotted (errors bars represent ± 1 std. dev.). (B) The glucose levels were grouped together as single samples, plotted (errors bars represent ± 1 std. dev.), and an *F*-test was performed on the group of samples. Additionally, a *t*-test was performed on the two samples exhibiting the lowest sensitivity—the highest glucose levels (20 and 15 mM). All *P* values showed a high statistical difference between the samples, indicating successful sensing of glucose.

to 20 mM (Fig. 5). The sensor was allowed to equilibrate in each glucose solution for 15 min, with data collected and averaged during the last 3 min in each solution; during this experiment, data were collected every 30 s, to obtain 6 data points for each glucose level ($n = 6$). The samples (glucose levels) were then grouped, and an *F*-test performed. The resulting *P* value was much lower than 0.001, showing strong evidence that the response of the sensor to different concentrations of glucose was statistically distinct. To take a more rigorous approach, a *t*-test was performed using the two least sensitive samples (20 and 15 mM), and again the *P* value was found to be much lower than 0.001, corroborating the statistical validity of the measurements.

To verify a lack of sensitivity to fructose, a fiber-optic GRP hydrogel sensor was exposed to solutions of 10 mM buffered glucose with fructose (a high amount at 0.5 mM) and without (Fig. 6); the experiment was carried out in the same manner as above. A *t*-test was performed on the two groups, this time finding a *P* value greater than 0.7, providing evidence that the two samples are statistically identical and that no sensing of fructose occurred.

With excellent glucose-response behavior displayed in buffered glucose solutions, *in vitro* testing was then carried out to validate the GRP sensor's function in whole porcine blood. A fiber-optic GRP hydrogel sensor was exposed to blood-glucose levels that represent high (hyperglycemia, 20 mM), low (hypoglycemia, 2 mM), and intermediate (10 mM) glucose levels present in humans (Fig. 7). As with the earlier tests, the sensor was allowed to equilibrate in each

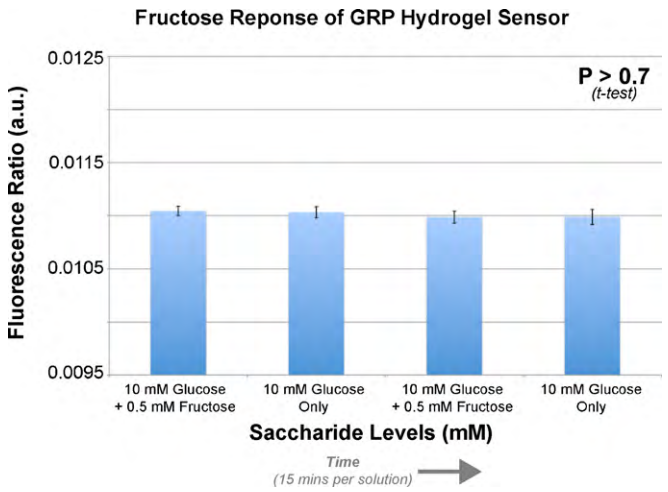

Fig. 6. Fructose response of a GRP hydrogel sensor. The sensor was exposed to buffered glucose solutions with and without high amounts of fructose. The sensor was allowed to stabilize in each solution for 12 min, data were taken for 3 min, and the data were then averaged and plotted (errors bars represent ± 1 std. dev.). Next, a *t*-test was performed on the two samples (with and without fructose). The resulting *P* value showed a very low statistical difference between the samples, indicating insensitivity to fructose.

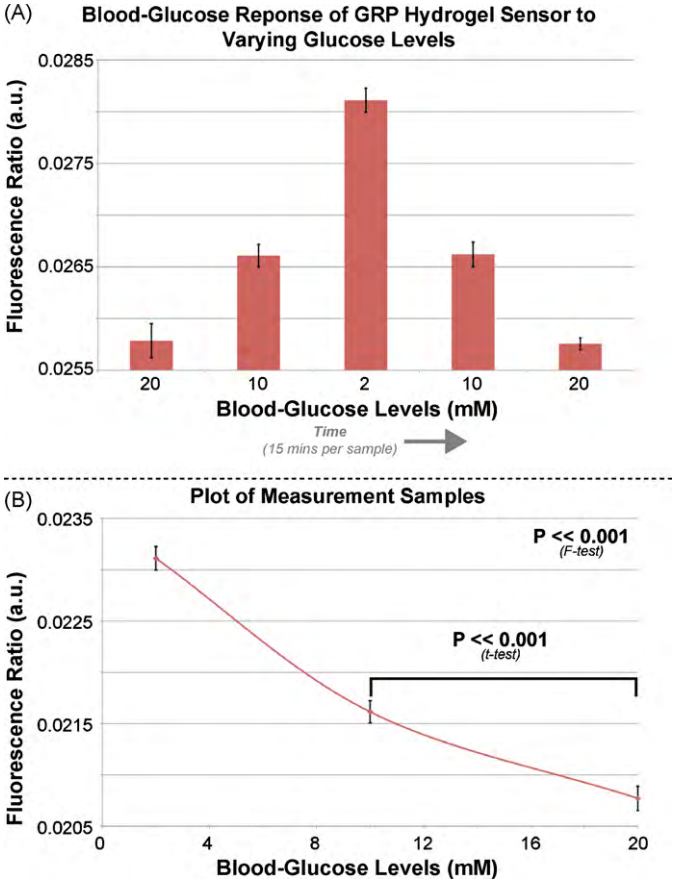

Fig. 7. Blood-glucose response of a GRP hydrogel sensor. The sensor was exposed to porcine blood spiked with varying saline glucose solutions. The sensor was allowed to stabilize in each blood sample for 12 min and then data were taken for 3 min. (A) The data were averaged and plotted (errors bars represent ± 1 std. dev.). (B) The glucose levels were grouped together as single samples, plotted (errors bars represent ± 1 std. dev.), and an *F*-test was performed on the group of samples. Additionally, a *t*-test was performed on the two samples exhibiting the lowest sensitivity—the highest glucose levels (20 and 10 mM). All *P* values showed a high statistical difference between the blood-glucose samples, indicating successful sensing of glucose.

blood-glucose solution for 15 min, with data collected and averaged during the last 3 min in each solution; data were collected every 30 s to obtain 6 data points for each blood-glucose level ($n = 6$). The samples (blood-glucose levels) were then grouped, and an *F*-test performed. The resulting *P* value was much lower than 0.001, showing strong evidence that the response of the sensor to different concentrations of glucose was statistically distinct. To take a more rigorous approach, a *t*-test was performed using the two least sensitive samples (20 and 10 mM glucose), and again the *P* value was found to be much lower than 0.001, corroborating the statistical validity of the measurements.

4. Discussion

4.1. Experimental discussion

Genetically engineered, fluorophore-labeled GRPs were successfully utilized in a fiber-optic based glucose biosensing system. After engineering, expression, and labeling of GRP, the functional polypeptide sensing elements were immobilized in a polyacrylamide hydrogel on the tip of a plastic optical fiber; this enabled the realization of a continuous glucose-sensing device by monitoring fluorescence intensity as a direct indicator of glucose concentration, and represents the first report in the literature of successful immobilization of functioning glucose binding protein-like sensing elements in a device.

Attachment of the hydrogel to the POF was adequate during *in vitro* testing and handling, but can be further enhanced through a covalent attachment scheme. The hydrogel chemistry itself was preliminarily optimized (data not shown), but through further optimization, enhancement in sensor performance likely will be obtained. Finally, the fiber-optic hardware system provided adequate performance for the purposes of this work, but could also benefit from further optimization, namely the inclusion of a more stable and specific light source such as an LED or laser diode.

From above, the results of the first experiment (Fig. 4) in buffered glucose solutions showed the response time of the sensors to be 10 min (even when moving from high to low glucose levels), with good signal stability (low sensor drift) and repeatability; the response time can be improved through engineering of the hydrogel geometry to increase the surface area-to-volume ratio and lower the diffusion equilibrium time. The GRP sensor also displayed a higher fluorescence intensity at lower glucose levels, displayed reversibility, and retained reliable and stable function for the duration of a continuous 1 h test.

The next experiment demonstrated excellent sensor signal reproducibility, stability, and functionality for the duration of the continuous 2.25 h test, showing that, for example, two data points corresponding to a 10 mM glucose reading taken 1 h apart are statistically identical after several cycles of exposing the sensor to varying glucose levels (Fig. 5). It is important to note from the excellent signal stability (indicated by small relative error bars) that the response time for equilibrium between the glucose levels is well under 12 min, consistent with earlier findings. These results also demonstrate a very important sensing characteristic of the GRP system: a non-linear glucose response that results in high sensitivity at low glucose levels. This sensor characteristic affords higher sensitivity in the hypoglycemic range, which is in stark contrast to any glucose-sensing system available today. The calculated *P* values ($P \ll 0.001$) further indicate strong statistical differentiation between the various glucose levels, including the areas where sensitivity is lowest (in the high glucose levels). Thus, glucose sensing and differentiation were successful using the prototype sensor.

Further appreciation for the hypoglycemic sensitivity can be gained by examining the precision displayed by the sensor. An average %CV of 0.3% was obtained during this experiment. Using a linear glucose-response approximation between each set of glucose levels, and assuming a precision of 1 standard deviation (std. dev.), the approximate precision displayed between the 90 mg/dL (5 mM) and 36 mg/dL (2 mM) levels is 3.6 mg/dL (0.2 mM), which is excellent for this prototype sensor. Following a 3-sigma precision (*i.e.*, assuming a precision of 3 std. devs.), the precision is just over 10 mg/dL. The same calculation between the 360 mg/dL (20 mM) and 270 mg/dL (15 mM) levels finds an approximate 1-sigma precision of 22 mg/dL and a 3-sigma precision of 65 mg/dL. However, when measuring blood-glucose levels in critical patients, accuracy in the low glucose levels is much more crucial than in the high glucose levels, where the former can cause hypoglycemic coma and other complications, with the latter causing more long-term but less acutely severe conditions.

The next experiment carried out was to determine the effects of fructose as a possible interfering sugar. For this GRP system, another relevant sugar is galactose, which can be present in blood at concentrations up to 0.1 mM. However, this is most clinically relevant for newborns, which exhibit higher blood-galactose levels. While there are many other different molecules that could potentially interfere with the glucose-sensing signal, fructose was chosen here because it can exhibit large dynamic changes dependent on, for example, a patient's diet. In this experiment, the results of the fructose sensitivity testing displayed a very stable fluorescence intensity with no statistical change when exposed to fructose (Fig. 6). The calculated *P* value was very high ($P > 0.7$), verifying all measurements (with and without fructose) provided a statistically equivalent value. Thus, the GRP sensor retains its high selectivity, and fructose will likely not have an impact on precision or accuracy of the CGM system.

Finally, the fiber-optic glucose sensor showed similar performance characteristics over the continuous 1.5 h test in blood as the sensor tested in buffered solutions (Fig. 7). Again, the sensor exhibited excellent sensor signal reproducibility, stability (low sensor drift), and functionality, as verified by the analysis of variance ($P \ll 0.001$). Performing the same gross linear glucose-response precision approximation from above, this time between the 36 mg/dL (2 mM) and 180 mg/dL (10 mM) levels, one finds a 1-sigma precision of 9.5 mg/dL. Moreover, this sensor was tested in porcine blood heated to 37 °C, verifying GRP's ability to continue functioning reliably at physiologic temperatures in complex solutions.

4.2. Advantages and applications

The GRP fiber-optic sensing system introduced here displays many advantages as a glucose sensor, namely high hypoglycemic sensitivity, high overall precision, and low interference from fructose. In addition, the sensor retains function between room temperature and physiologic temperature. These characteristics translate to low sensor drift and the ability to better retain calibration when used in a continuous, *in vivo* environment, thus promising high accuracy. Although an extensive lifetime study was not presented in this work, the GRP hydrogel sensors were found to retain function for many months (data not shown). For example, the sensor used in the experiment from Fig. 4 was approximately 2 months old at the time of data acquisition, and had been used repeatedly for prior testing.

This GRP sensing system has many potential applications, especially as an invasive device for TGC in critical care wards. The plastic fiber-optic with attached hydrogel GRP sensor can be used as a disposable catheter and inserted intravenously to continuously monitor blood glucose at the bedside; the fiber-optic measurement system could be easily miniaturized for bedside use. Such a device would enable TGC to be performed without the worry of high error in the hypoglycemic ranges, something that hinders implementation of TGC today. Moreover, the *in vivo* lifetime requirement

of a TGC sensor is on the order of days (not months), easing the requirement of retained accuracy. If coupled with an insulin delivery system, an artificial pancreas system could also be realized. Finally, the GRP sensor could also find applications in the home market as an upgraded sensor in today's handheld POC glucometers or as a replacement to the currently unreliable subcutaneous CGM systems.

4.3. Limitations and future work

While the GRP system holds much promise as a continuous glucose monitoring technology, it, like any other system, does not come without limitations. Namely, the system relies on the measurement and tracking of fluorescence intensities, rather than wavelength shifts or frequency-domain measurements. Reliable intensity measurements can be difficult, and require the appropriate hardware with optical control of the sensor environment. Reproducible sensor-to-sensor fabrication was difficult in this study, although can be greatly improved through a controlled manufacturing environment.

There is much work to be done in the continued advancement of this system, and some of the future tasks required will be briefly highlighted here. As related to engineering aspects of the system, the physical stability and durability of the sensor will need to be characterized and optimized, and sensor dimensions (*e.g.*, fiber-optic diameter) will need to be optimized to achieve ideal optical behavior. As mentioned above, alternate, compact light sources such as LEDs and laser diodes will need to be tested. As with any indwelling *in vivo* sensor, fibrous encapsulation and protein adsorption can hinder functionality; however, the GRP system promises an extremely high starting accuracy. Regardless, extensive studies will need to be performed to characterize sensor drift and changes in response time as related to sensor biofouling. Moreover, systems will need to be developed to enable placement of this sensor *in vivo* into a vein or artery with optimized positioning such that sensing is uninhibited and the aforementioned sensor biofouling is minimized. Additional interfering molecules will also need to be analyzed, including other sugars such as sucrose, xylose, maltose, and galactose, discussed above. Extensive validation studies will need to be performed to compare multiple sensors at many glucose levels over long periods of time (days to months), such that calibration procedures (*e.g.*, calibration transfer, calibration maintenance, etc.) can be developed. Finally, the packaging and distribution of such a fluorescent hydrogel-based GRP sensor will need to be studied, and would likely require storage under dark and liquid conditions.

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Disclosure statement

For full disclosure and conflict-of-interest transparency, it should be noted that J. Siegrist and T. Kazarian were employees/consultants for iGlyko, Inc. at the time of this work and publication preparation. They are, however, not currently iGlyko employees. It should also be noted that three of the other authors (M. Madou, P. Wang, and S. Daunert) are scientific advisors and shareholders of iGlyko, Inc. As indicated in the text of the manuscript, the work presented is related to a patent application [40]. However, while iGlyko is licensing this intellectual property, it is owned by the University of Kentucky.

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