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Fluorophore-labeled bioengineered glucose binding protein for measurement of transdermal glucose

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Measurement of glucose is an important component of care in the intensive care unit. Current methodologies for glucose monitoring include enzymatic-based laboratory analyzers, point of care testing and continuous glucose monitoring systems that all require breaking the skin. This study focuses on the development of a glucose biosensor to measure transdermal glucose (TG) by noninvasive passive diffusion. The H152C glucose binding protein (GBP) labeled with the polarity-sensitive dye BADAN (6-bromoacetyl-2-dimethylaminonaphthalene) was prepared and characterized with the aim of producing a biosensor with an operating range at micromolar levels compatible with TG concentrations. The BADAN-labeled GBP exhibited a large fluorescence intensity increase (228%) upon addition of a saturating concentration of glucose. The glucose binding constant was calculated to be (K_d) 1.124 ± 0.2361 μM. The biosensor has a linear operating range of 0.030–0.460 μM, making it suitable for monitoring transdermal glucose. The use of the biosensor was demonstrated by measuring TG collected by passive diffusion of glucose through the skin of healthy adult subjects. Results showed that the H152C GBP labeled with BADAN is capable of measuring TG and can be used for noninvasive glucose sensing.

Keywords: glucose binding protein, biosensor, BADAN, transdermal glucose

Introduction

Current FDA-approved blood glucose devices are based on biocatalytic elements such as glucose oxidase [1–5], glucose dehydrogenase [6] and hexokinase [7]. An alternative technology for glucose sensing employs bioaffinity elements such as the glucose binding protein (GBP) [8–13]. GBP is a member of a diverse group of transport proteins from Gram-negative bacteria such as *E. coli*. Unlike enzymes, GBP does not require other reactants and co-factors. It exhibits high binding sensitivity, specificity and affinity to glucose. The binding equilibrium is straightforward, simple, reversible, and generates no reactive by-products. Signal transduction arises from a change in conformation of the protein upon binding with glucose, and could be quantified through a polarity-sensitive fluorescent dye, which can differentiate the conformations of GBP.

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The binding constant (K_d) of GBP for glucose is in the micromolar level, making GBP suitable for the detection of low glucose concentrations such as in transdermal glucose flux in permeated skins. The capability of GBP to measure transdermal glucose has been demonstrated [14, 15]. Transdermal glucose (TG) measurement has gained interests due to its noninvasiveness in nature and its potential in medical diagnosis on challenged patients.

Efforts have been made to increase the binding constant to millimolar levels by studying different mutants of GBP to enable methods that can complement blood glucose levels [16, 17]. A GBP mutant (L255C) had been applied for measurement of TG [14, 15]. In this paper, a bioengineered GBP (H152C) was prepared and characterized as a recognition element for TG measurement. This mutant has a polyhistidine tag that allows for quicker purification and provides a handle for protein immobilization.

Experimental

Materials. D-Glucose, NaH_2PO_4, Na_2HPO_4, $NaH_2PO_4.H_2O$, N,N-dimethylformamide, KH_2PO_4 and Coomassie Brilliant Blue solution were acquired from Sigma-Aldrich (St. Louis, MO). BADAN (6-bromoacetyl-2-dimethylamino-naphthalene) was obtained from Molecular Probes (Eugene, OR). Slide-A-lyzer dialysis casettes were obtained from Thermo Scientific (Rockford, IL). Sephadex G-25 fine were from GE Healthcare (Bio Sciences, AB). Luria-Bertani (LB) medium, ampicillin, isopropyl-2-D-thiogalactopyranoside (IPTG), guanidium chloride (GuHCl), Tris-hydrochloride and urea were from Fisher Scientific. LB medium supplemented by antibiotics (100 μg/mL ampicillin) was used to allow growth of cells. His Pur Ni-NTA resin and tris(2-carboxyethyl) phosphine (TCEP) were obtained from Thermo Scientific (Rockford, IL). All chemicals were used without further purification. Plasmids were created and purchased from Life Technologies.

Modified *E. coli* (BL21 DE3 strain) containing the plasmid (GBP H152C-pET303 CT His) was used as source of GBP. It was constructed in the laboratory by molecular cloning of *E. coli* (BL21 DE3 strain). Pre-constructed mutant (GBP H152C) was suspended in water and underwent transformation with BL 21 competent cells. Clones were taken for the mutant and glycerol stocks were made and inoculated.

Preparation of H152C GBP mutant. The H152C GBP was expressed, separated and purified as described previously [10, 11, 13] with some modifications. The GBP was overexpressed at the periplasmic space by growing the modified *E. coli* cells in LB media. The cell growth was measured at the end of 3 h by determining its optical density at 600 nm using the spectrophotometer (Agilent technologies). At 0.4 optical density, expression of GBP was induced by adding 500 μL of 1 M isopropyl-2-D-thiogalactopyranoside (IPTG) and grown for 4–5 h at 37°C at 225 rpm agitation.

Bacterial cells were lysed through sonication for 10 min in a beaker containing ice using the probe sonicator (Fisher Scientific Sonic Dismembrator Model 500). Affinity chromatography was performed in two columns packed with 5 mL of equilibrated Ni-NTA agarose resin (Thermo Scientific).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify the size, presence and purity of GBP. The concentration of the total protein of the isolated GBP was determined using Bradford Protein Assay. The highly pure fractions were dialysed using 20 mM phosphate (NaH_2PO_4 and Na_2HPO_4) buffer, pH 7.4 at 4°C.

The GBP was labeled with environmentally sensitive fluorophore, BADAN at the single cysteine mutation at position 152 following the manufacturer's recommended labeling procedure. The fluorophore was covalently

attached to the cysteine mutation and known to be responsive to glucose [11, 16]. Unreacted BADAN was removed by size exclusion chromatography column, Sephadex G-25 fine resin. The extent of labeling was determined from the GBP concentration and optical density of protein with dye. The following equation was used for the calculation of the extent of labeling (1):

$$Extent\ of\ labelling = \frac{mole\ dye}{mole\ protein} * 100 = \frac{Ax}{\varepsilon} * \frac{MWprotein}{protein\ conc\ (\frac{mg}{mL})} * 100$$

where A_x is the absorbance value of the dye, BADAN at the absorption maximum wavelength, ε is the molar extinction coefficient of the dye at the absorption maximum, which is 20000 and MW is the molecular weight of protein, which is 33310 g/mol.

The purified protein was dialysed in 100 mM phosphate buffer, pH 7.5. The final product was 0.22 μm filter-sterilized and stored at 4°C.

Fluorescence measurements. The fluorescence intensities of BADAN-labeled GBP in the absence and presence of D-glucose in 1× PBS buffer were measured on a 96-well SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). BADAN was excited at 400 nm, with the maximum fluorescence emission (Em_{max}) at 550 nm. 200 μL of GBP solution was added to each of the designated wells. Glucose standards or samples (5.0 μL) were then added to each of the wells. The plate was gently shaken for 5 s, and the intensities were measured 4 times. All measurements were made following the same instrumental conditions: excitation wavelength 400 nm, emission wavelength 550 nm, excitation and emission slit width 5 nm, PMT detector voltage 750 V, and average time 0.1 s.

Standard solutions were prepared by dissolving $_D$-glucose (>99.5% purity) from Sigma-Aldrich (St. Louis, MO) in 1× PBS, pH 7.4. A 1 mM glucose solution, pH 7.5 was used as a stock solution. The stock solution was then diluted to the following concentrations: 1, 2, 4, 6, 8, 10, 20, 50, 100, and 200 μM. Fluorescence intensities of GBP in the presence of glucose were plotted against glucose concentration (μM) to attain the calibration curve.

The mean fluorescence readings were calculated from the four readings. Measurements are reported as normalized fluorescence intensity calculated as:

$$\frac{(F - Fo)}{Fo}$$

where the F and F_0 were the fluorescence intensities of GBP in the presence and absence of glucose, respectively.

The Varian Cary Eclipse fluorescence spectrophotometer was used to measure the fluorescence spectra. BADAN was excited at 390 nm with a slit width of 5 nm. The emission spectra were measured in the range of 400–650 nm with the maximum fluorescence emission (Em_{max}) occurred approximately at 540 nm, using the following conditions: high PMT detector voltage, average read time 0.10 s, excitation filter auto and emission filter open.

Reusability (Reversibility) of the glucose binding protein. To check the possibility of BADAN-labeled GBP as a reversible biosensor, previously used GBP was recovered after the assay and studied for its reusability. To remove the bound glucose, the used GBP solution was injected into a Slide-A-lyzer dialysis cassette, which was then dialyzed in 1× PBS buffer for 6 h in a cold room. Afterwards, the 1× PBS buffer was replaced with fresh buffer and the GBP was dialyzed for another 6 h. The GBP solution was transferred from the dialysis cassette to a sterile glass vial and filtered-sterilized. The recovered GBP solution was tested with glucose standard solution and the results were compared with the fresh GBP solution. The dialysis and recovery steps were repeated several times to see whether the GBP response remains the same.

Determination of the binding constant of GBP. The binding constant K_d of the GBP and its maximum glucose binding were calculated by fitting the data of fluorescence intensities (a.u.) at different glucose concentrations (μM) to binding-saturation curves using Prism 6 (GraphPad Software, San Diego, CA, USA).

Measurement of transdermal glucose. Samples for transdermal glucose measurement were collected from healthy adult subjects, specifically from the skin of the fingers. The sampling protocol involved washing the hands with soap and water and followed by a distilled water rinse. The finger was then submerged in an agitated 20 mM phosphate buffer in a beaker for 5 min. The buffer was kept at constant temperature of 37°C using a jacketed beaker and continuous circulating water bath. Buffer temperature was monitored using an electronic thermometer. The washing step was followed by drying of the finger for 2 min with filtered air. Washing and drying steps are important to remove the residual glucose from the stratum corneum. This was to ensure that the collected sample is the glucose diffusing through the skin at time of collection. The five-minute increased temperature wash was also optimized [20].

Collection of the transdermal glucose sample was done by inverting a 1.5 mL Eppendorf tube containing 250 μL of 20 mM phosphate buffer at room temperature on the same finger for 5 min. The total sampling time was 12 min. The sample was collected and stored at –80°C. About 20 μL TG samples were added to 200 μL of the GBP solution. The fluorescence intensities were measured as previously described for the glucose standard solutions and the change in intensities were calculated against GBP without samples. The TG concentrations in the samples were calculated from the calibration curve.

To induce larger blood glucose changes, subjects were subjected to an oral glucose tolerance test. Samples for transdermal glucose measurement were collected from healthy adult subjects who had been fasting overnight. Two TG samples were collected during the fasting stage at about 30 min interval. After taking 75 g glucose, samples were then collected from the same subjects at 30 min time intervals for 2 h. These steps capture the increase and steady decrease of glucose levels in the subject's body after consuming glucose.

Measurement of blood glucose. The blood glucose (BG) levels of the subjects were measured concurrently with the transdermal glucose. Corresponding BG levels were determined using a commercially available glucometer (ReliOn™ Confirm glucose meter). The BG level was measured on the same finger where TG was collected.

Results and Discussion

H152C GBP preparation. The H152C mutant of the glucose binding protein used in the experiments contained a polyhistidine tag, which allows for ease of purification. It was isolated from the lysate of the bacterial cells of modified *E. coli* by means of sonication and affinity chromatography. The SDS-PAGE of the purified GBP showed two thick bands at the 34 kDa position consistent with the size of the GBP [8] (Fig. 1).

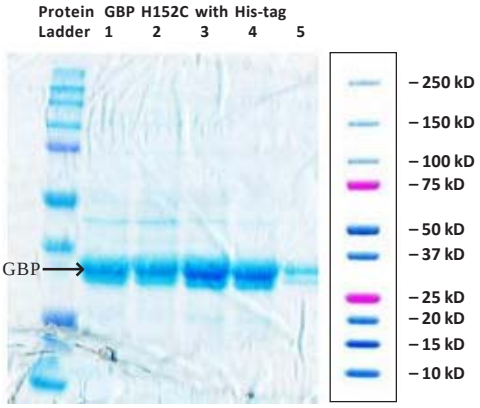

Figure 1. SDS-PAGE results showing the presence of H152C GBP

The protein had a total concentration of 8.5 μM and the calculated labeling extent of protein-bound BADAN was found to be 83.17%.

Fluorescence of BADAN-labeled GBP. When the GBP labeled with BADAN was excited at 400 nm, the maximum emission spectra occurred at approximately 540 nm. Addition of saturating concentration of glucose (42 mM) to GBP resulted in an increase in fluorescence intensity of 228%. Figure 2 shows the change in the fluorescence spectra of the protein in the absence and in the presence of glucose.

BADAN, a polarity sensitive dye, was reacted to the cysteine mutation at position 152 near the glucose-binding site in GBP. The observed increase in fluorescence intensity upon binding with glucose is due to the increased hydrophobicity around this site brought about by the closing of the two globular domains of the protein around glucose [18].

The fluorescence intensity of the BADAN-labeled GBP increased with increasing glucose concentrations, varying significantly at low concentrations and only slightly at the higher concentrations of 0.674 and 1.085 μM glucose (Fig. 3). A linear behavior occurred at the concentration range of 0.030–0.460 μM (combine Fig. 4 and Fig. 5). Within this range, the GBP displayed good sensitivity (1.68 μM^{-1}) to glucose concentration and is most useful for

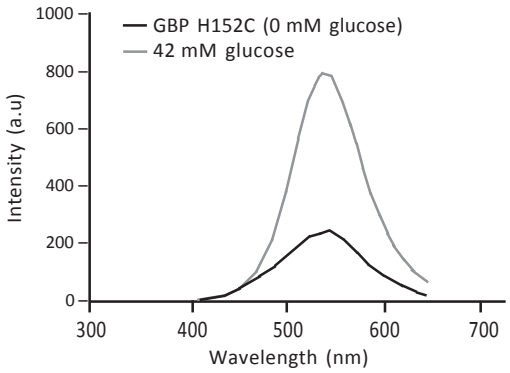

Figure 2. Emission spectra of GBP labeled with BADAN showing the increase in fluorescence intensity upon addition of glucose

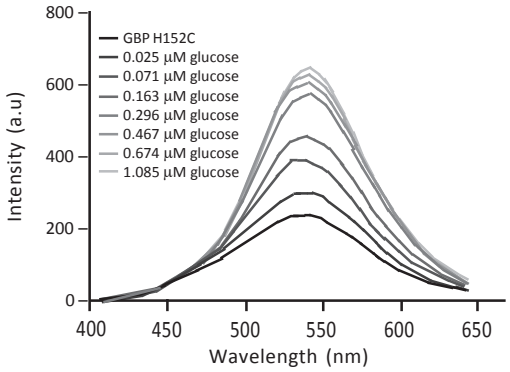

Figure 3. Emission spectra of BADAN-labeled GBP with increasing glucose concentrations

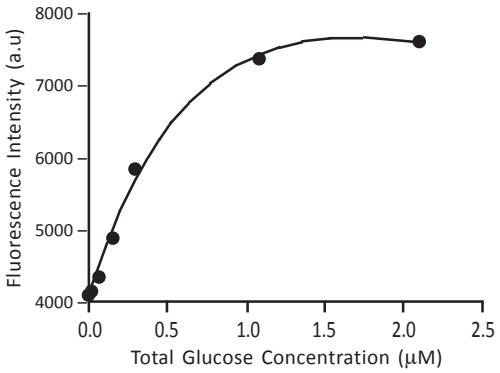

Figure 4. Fluorescence intensity of GBP as a function of total glucose concentration (μM) (n = 4)

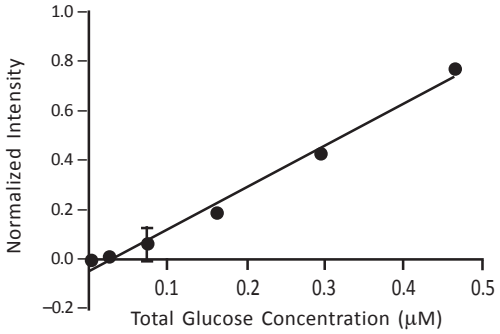

Figure 5. Linear range of calibration curve (n = 4)

glucose measurement. The limit of detection of the assay (based on 3 s) is 0.030 μM.

Binding constant of the GBP and calibration curve. The binding constant, K_d of GBP H152C-BADAN was found to be 1.124±0.236 μM (Fig. 4). The apparent K_d was slightly lower compared from previous work, 2.35 mM [18]. The binding constant, can be calculated by fitting experimental results from GBP response corresponding to different glucose concentrations to the binding isotherm (2):

$$\Delta F = \frac{\Delta Fmax\,[S]}{(Kd + [S])}$$

where ΔF is the normalized signal change at any ligand concentration, ΔF_{max} is the normalized signal change at saturating ligand concentration, [S] is the concentration of the ligand in free state and K_d is the binding constant. This equation along with equations for single binding equilibria and mass balance produce the following equation (3):

$$\frac{\Delta Fmax}{\Delta F} = 1 + \frac{Kd}{[S]t - \frac{\Delta F}{\Delta Fmax}[E]t}$$

where $[E]_t$ and $[S]_t$ are the total concentrations of GBP and glucose respectively. The experimental results can be analyzed with nonlinear regression and the best values for "F_{max} and K_d can be determined.

Reusability of the glucose binding protein. Reusability tests showed that the calibration curves did not change significantly after several recoveries (Fig. 6). These tests only proved that the GBP could be used many times, which could be considered as a potential reversible and reusable sensor [19].

Transdermal glucose measurement. Prior to the collection of TG sample, the finger was subjected to washing and drying in order to remove the existing glucose in the stratum corneum. These steps ensure that the collected glucose is the TG diffusing through the skin. Previous study showed that the temperature of the washing buffer and drying step are important parameters. An increase in temperature of washing buffer decreased the washing time required - from an initially 15-min wash at room temperature [15] to 5-min at 37°C [20]. Table 1 shows the optimized parameters for sampling and analysis.

The TG concentrations in the collected samples were determined from a previously prepared calibration curve (Fig. 5). The results of the TG measurements for samples collected at different times during fasting and after intake of glucose are shown in Fig. 7 together with the results of BG measurements. It could be seen that TG levels follow the same trend as the BG. However, TG concentration had an average lag time of 49 min for individuals 1 and 2. A peak value of

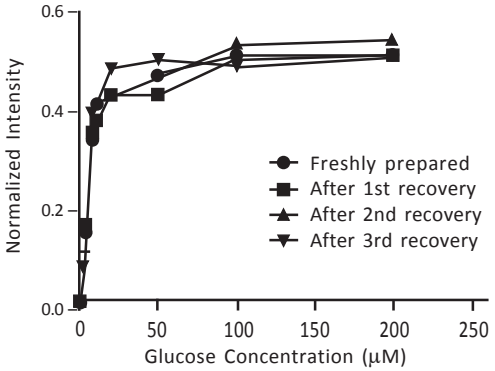

Figure 6. Reusability test of the BADAN-labeled H152C GBP (n = 4)

Table 1. Optimized Parameters for Noninvasive Transdermal Glucose Sampling and Analysis

Parameter	*Optimized value*
Wash time	5 min [20]
PBS Buffer temperature	37°C [20]
Drying time	2 min [15]
Sampling time	5 min [15]
Volume of PBS buffer	250 μL [14, 15]
Volume of GBP	200 μL

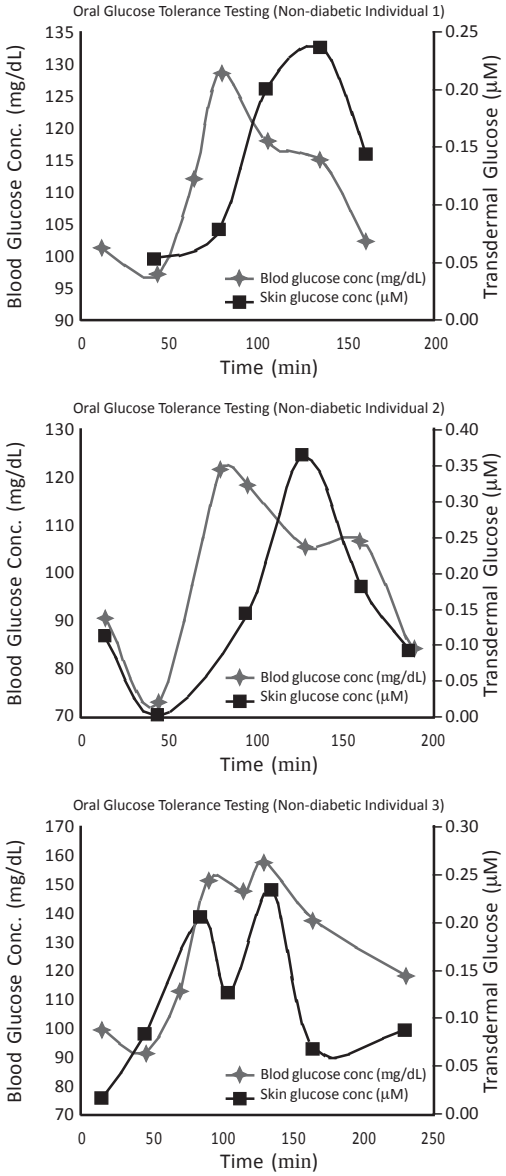

Figure 7. Comparison of TG and BG levels of healthy adult subjects from their Oral Glucose Tolerance Tests (OGTT) (Individuals 1–3)

0.24–0.37 μM were observed for the TG concentrations and 122–152 for the BG levels. A lag is expected in skin glucose samples because of the time required for ingested glucose to circulate to the blood, the tissues and then through the skin. Further investigation is needed to account for the lag time. However, the results for individual 3 did not show a lag time, which can be associated with the age of individual. These results showed a correlation exists between TG and BG.

Conclusion

A BADAN-labeled mutant of GBP (H152C) was synthesized, prepared and characterized. It had a K_d in the micromolar levels indicating its capability to detect low TG concentrations. Fluorescence intensities increased markedly upon addition of glucose. There is a correlation between the measured transdermal glucose and blood glucose levels with a lag of TG behind blood glucose.

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