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A Glucose Sensor Protein for Continuous Glucose Monitoring

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Abstract

In vivo continuous glucose monitoring has posed a significant challenge to glucose sensor development due to the lack of reliable techniques that are non-or at least minimally-invasive. In this proof-of-concept study, we demonstrated the development of a new glucose sensor protein, AcGFP1-GBPcys-mCherry, and an optical sensor assembly, capable of generating quantifiable FRET (fluorescence resonance energy transfer) signals for glucose monitoring. Our experimental data showed that the engineered glucose sensor protein can generate measureable FRET signals in response to glucose concentrations varying from 25 to 800 μ M. The sensor developed based on this protein had a shelf life of up to 3 weeks. The sensor response was devoid of interference from compounds like galactose, fructose, lactose, mannose, and mannitol when tested at physiologically significant concentrations of these compounds. This new glucose sensor protein can potentially be used to develop implantable glucose sensors for continuous glucose monitoring.

Keywords

Glucose sensor; FRET molecular probe; continuous glucose monitoring; glucose binding protein; biosensor; glucose indicator protein

1. Introduction

Effective diabetes management relies upon glucose monitoring, which is currently performed in a painful and inconvenient finger-prick blood glucose testing fashion. However, glucose monitoring in body fluids has become more attractive to diabetes management due to its potential for achieving continuous glucose monitoring (CGM) through non-or minimally-invasive detection. A body of evidence suggests that diabetes management can significantly benefit from CGM (Edelman and Bailey 2009; Gilliam and Hirsch 2009). CGM can also be coupled with an insulin delivery pump, forming a closed-loop insulin delivery system to achieve optimal insulin delivery all the time for maintaining blood glucose at physiological levels all the time (Mastrototaro and Lee 2009).

In the past decades, CGM has always been performed using body fluids, such as interstitial fluids, through an implanted glucose sensor. For example, an implantable electrometric glucose sensor has been developed and used for continuous monitoring glucose concentrations in body interstitial fluids (McGarraugh 2009). The localized glucose depletion and voltage applied to the tissues by this type of sensor have been shown to lead to skin irritation in certain patients (Edelman and Bailey 2009; McGarraugh 2009; Wadwa et al. 2009). While a number of other types of CGM sensors have been developed (Chih Liao et al. 2004; Pasic et al. 2007), they all rely upon the implantation of sensors beneath either

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skins or other tissues such as subcutaneous tissues (Chih Liao et al. 2004; McGarraugh 2009; Pasic et al. 2007). Nevertheless, these sensors are invasive to the body. Thus, it is of critical importance to develop a new technology for noninvasive glucose monitoring.

Studies revealed that the glucose concentration in body fluids such as tear and saliva are correlated with blood glucose (Iguchi et al. 2007; Yamaguchi et al. 1998). Monitoring glucose in these body fluids can potentially be achieved noninvasively. For example, a glucose sensor can be implanted inside a tooth or be integrated into a contact lens to continuously monitor glucose concentration in saliva or tear. The glucose concentration in saliva has been found to be in a micromole range from 8 to 210 μM (Yamaguchi et al. 1998), while glucose concentration in human tears is in the range from 128 to 166 μM (Chen et al. 1996; Jin et al. 1997; Mitsubayashi et al. 2003). Currently, commercially available glucose sensors are unable to detect such low concentrations directly. Although a sensor developed by Macaya *et al.* can detect glucose from 10 μM to 1 mM, it is not appropriate for CGM in saliva, as it requires the addition of enzymes to the reaction solution in order to increase the sensitivity of the sensor (Macaya et al. 2007). Currently, the glucose concentration in these body fluids is determined using mass spectrometer, which is unsuitable for CGM (Taormina et al. 2007).

To meet these needs, we engineered a new glucose responsive protein (Garrett et al. 2008; Ye and Schultz 2003). The glucose sensing ability of the protein was established by introducing a FRET (fluorescence resonance energy transfer) signal transduction mechanism directly into a glucose binding protein (GBP) in such a manner that the binding of glucose to the protein generates FRET signals for CGM through ratiometric FRET measurement (Garrett et al. 2008; Ye and Schultz 2003). To further adjust the glucose response of the protein to a range that covers glucose levels in body fluids, we modified the protein structure through site-directed mutagenesis (Jin, *et. al. personal communication*). Using this new protein, we developed a sensor prototype capable of monitoring glucose in a micromole range.

2. Materials and methods

2.1. Plasmid

The plasmid encoding the sensor protein, referred to as AcGFP1-GBPcys-mCherry, was constructed as follows: The genes encoding the AcGFP1 and mCherry were PCR-amplified from pAcGFP1 and pmCherry-N1 vectors (Clontech, Mountain view, CA), respectively, using the following pairs of primers: AcGFP1-forward primer: 5' ACACCGACTCTAGCTAGAGGATCT 3', AcGFP1-reverse primer: 5' ATCAGCTCCGACTTGTACAGCTC 3', mCherry-forward primer: 5' ATTTAAGAATTCAGCAAGAAAATGGTGAGCAAGGGCGAG 3', mCherry-reverse primer: 5' TACTTGAAGCTTGCTTGTACAGCTCGTCCATG 3'. The mutated GBP, GBPcys (F16C) was developed at our lab previously (Jin, personal communication). The gene encoding the GBP mutant was amplified using following primers: (forward primer) 5' ATGGATGAGCTGTACAAGTCCGAGCTGATACTCGCATTGGTGTAAACA 3' and (reverse primer) 5' TACTTGAAGCTTGTTTCTTGCTGAA 3'. The resultant AcGFP1 and GBPcys PCR fragments were subjected to an overlapped PCR extension to create an AcGFP1-GBPcys fusion fragment. This donor-only gene was then sub-cloned into pTA/GBP backbone (Ye and Schultz 2003) through NcoI and HindIII sites. The mCherry gene was sub-cloned in the downstream of AcGFP1-GBPcys fusion gene through EcoRI and HindIII sites, leading to the expression of the sensor protein AcGFP1-GBPcys-mCherry from the *trc* promoter (Ye and Schultz 2003). A(His)₆ tag was fused at the C-terminus of the fusion protein to facilitate the protein purification through single-step IMAC (immobilized metal affinity chromatography) purification.

2.2. Protein expression and purification

pTA-AcGFP1-GBPcys-mCherry was transformed into the *E. coli* DH5 α (F- ϕ 80dlacZ Δ M15 Δ (lacZY-argF) U169 endA1 recA1 hsdR17 (rk-mk+) deoR thi-phoA supE44 λ -gyrA96 relA1). The recombinant *E. coli* was incubated at 37°C while shaking at 225 rpm for 24 h. The IPTG (Isopropyl β -D-1-thiogalactopyranoside)(final concentration: 1 mM) from Gold Biotechnology Inc. (St. Louis, MO) was used to induce the expression of AcGFP1-GBPcys-mCherry protein when the OD₆₀₀ of the cell cultures reached about 0.5–0.6. The recombinant *E. coli* was harvested by centrifugation at 3,200 rpm for 15 min, and re-suspended in a BPERII cell lysis buffer (Pierce Inc, IL) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich Inc, St. Louis, MO). Cell debris was removed by centrifugation at 15,000 rpm for 15 min. The cell-free extracts were stored at 4°C until use. A 5 ml HiTrap ChelatingHP affinity column (GE Healthcare Biosciences, Sweden) was assembled and used for IMAC purification of the protein, following the procedure provided by the manufacture. The proteins were eluted with 30 mL of elution buffer (20 mM Tris-HCl, 500 mM NaCl, and 500 mM imidazole, pH7.5) at a flow rate of 1 mL/min, and fractions of 1 ml each were collected using an automated fraction collector from Bio-Rad (Hercules, CA). The fractions with the highest OD₂₈₀ absorbance were collected and pooled together for glucose binding assays. The purified proteins were dialyzed against sugar-free dialysis buffer (20 mM Tris-HCl, 5 mM dithioerythritol, 150 mM NaCl, and 1 mM CaCl₂, pH7.5) at 4°C in a Float-A-Lyzer ready-to-use dialysis device (Spectrum Laboratories, Inc., Rancho Dominguez, CA) with a 25-kDa molecular weight cutoff in order to remove imidazole and any bound sugars from the proteins. The proteins were concentrated with the YM-30 ultrafiltration membrane (Millipore, MA), aliquoted, and stored at –20°C until use.

Protein concentrations were determined through the BCA protein assay (Stoscheck 1990) with bovine serum albumin (BSA) as a standard. The purity of the proteins was ascertained by SDS-PAGE, using a 12% polyacrylamide gel, and the protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (CBB R-250) and documented using the ChemiDoc XRS Gel documentation system (Bio-Rad, CA).

The sensor protein was characterized by titrating highly concentrated glucose into the protein solution, and the FRET signal is defined as the fluorescence intensity of AcGFP1 over mCherry in response to glucose concentration. It was measured by a two-wavelength measurement of AcGFP1 and mCherry's fluorescent intensities at 505 nm and 610 nm, respectively, when excited at 475 nm. The slit width was set at 10 nm for both excitation and emission. All the measurements were performed at room temperature.

2.3. Sensor Fabrication and CGM

The glucose microsensor was fabricated as follows: Purified AcGFP1-GBPcys-mCherry protein was loaded onto a dialysis hollow fiber made up of regenerated cellulose (Spectrum Laboratories, CA; molecular weight cut-off of 18 kDa with outer diameter: 216 μ m; inner diameter: 200 μ m; surface area/length: 6.3 mm²/cm; and volume/length: 0.31 μ L/cm). Proteins were evenly diffused into the hollow fiber membrane through capillary action, as described in our previous work (Ye and Schultz 2003). Then the upper end of the hollow fiber was sealed with cyanoacrylate (Henkel Consumer Adhesives Inc, OH). The hollow fiber segment was then removed from the solution and cut to a length of 1.5 cm, and the other end was also sealed with cyanoacrylate (3 μ g of total protein per sensor). The sensor was stored in a binding buffer (20 mM Tris-HCl, 5 mM dithioerythritol, 150 mM NaCl, and 1 mM CaCl₂, pH7.5) until use. The FRET signals were measured using the LS-55B Luminescence Spectrophotometer (Perkin-Elmer Instruments, Beaconsfield, UK). The hollow fiber membrane was placed inside a 7 μ L quartz cuvette, forming a flow cell unit.

Sugar-free binding buffer was perfused through the cell flow unit to yield a baseline, and varying concentrations of glucose were used to examine the response of the sensor to glucose. All of the buffers and assay solutions were flown through the flow cell unit, controlled by a constant flow syringe pump (KD scientific, Holliston, MA) at 0.33 ml/min.

For interference studies, solutions containing lactose, mannose, mannitol, galactose, and fructose (Fisher Scientific, PA) were prepared in the binding buffer and passed through the sensor, either as pure solutions or along with a known concentration of glucose.

All the measurements were carried out at room temperature in at least triplicate.

3. Results and discussions

3.1. Construction of a Micromole Glucose Sensor Protein for CGM

We have previously constructed a glucose indicator protein (GIP) for continuous glucose monitoring through ratiometric FRET measurement (Garrett et al. 2008; Ye and Schultz 2003). GIP was developed using a GBP with very strong affinity for glucose. Its dissociation constant, K_d for glucose is about 0.2 μM (Vyas et al. 1991); thus, the GIP developed using this GBP responds to glucose only in a sub-micromolar range (Ye and Schultz 2003). Furthermore, we showed that the working range of a FRET sensor is usually very narrow (Ye and Schultz 2003) due to the low efficiency of resonance energy transfer from a donor to its acceptor fluorescent protein used for the construction of a FRET sensor (Goedhart et al. 2007; Tramier et al. 2006). As mentioned above, glucose concentrations in some body fluids such as saliva and tear are in a micromolar range. It is, therefore, highly desired to develop a new glucose indicator or sensor protein capable of detecting glucose continuously in a micromolar range. To do so, we modified the GBP's molecular structure through site-directed mutagenesis, in which a cysteine residue was introduced at the 16th position of GBP's amino acid sequence. The replacement of phenylalanine with cysteine at its 16th position of amino acid sequence led to a significant reduction in its affinity for glucose. As shown in Fig. 1, the K_d value of mutant GBP increased up to 130 μM , which is suitable for detecting glucose concentration in body fluids such as tear and saliva.

We speculated that the introduction of cysteine at the glucose binding site of the GBP changes the binding characteristics of the GBP by steric effects, leading to the reduction of its affinity for glucose. For example, it was reported that the inclusion of a cysteine residue in a sulphate binding periplasmic protein resulted in 3,200 fold increase in the dissociation constant (He and Quijcho 1991). Structurally, GBP is very similar to the sulphate binding periplasmic protein. They all belong to a superfamily of periplasmic metabolite binding proteins (Ames 1986; Dwyer and Hellinga 2004).

Another modification that we made to improve the stability and sensitivity of the glucose sensor protein is to use a new FRET pair in place of the enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) FRET pair used in our early study (Garrett et al. 2008). As pointed out by a number of studies, the crosstalk or bleed-through between ECFP and EYFP can remarkably interfere with quantitative analysis performed using ECFP/EYFP pair-based FRET sensor (Erickson et al. 2003; Gordon et al. 1998; Rizzo et al. 2004). The strong photo bleaching of ECFP makes it virtually impossible to use ECFP/EYFP-based FRET sensors to continuously monitor biomolecules such as glucose. Fig. 2 illustrates a molecular structure of the AcGFP1/mCherry-based glucose sensor protein. AcGFP1 and mCherry were selected because of their high quantum yield and well separated excitation spectra (Tramier et al. 2006; van der Krogt et al. 2008). Their resistance to the photo bleaching is another factor that is superior to the ECFP/EYFP FRET pair for CGM.

3.2. Continuous Glucose Monitoring

3.2.1. Sensor Fabrication—A prototype of hollow fiber glucose sensor was developed using the AcGFP1-GBPcys-mCherry protein. The sensor was fabricated using a hollow fiber dialysis module. The sugar-free protein, AcGFP1-GBPcys-mCherry, was enclosed in a hollow fiber dialysis membrane, which serves as a barrier to entrap the protein while allowing the diffusion of glucose into the hollow fiber membrane to react with the protein. The proteins were evenly diffused into the hollow fiber membrane by dipping the membrane into the protein solution, as described in our previous work (Ye and Schultz 2003). No aggregation and precipitation of protein inside the hollow fiber membrane was observed. The entrapped AcGFP1-GBPcys-mCherry acts as a cognition element of the biosensor for glucose detection. The hollow fiber sensor was assembled in a continuous flow cell unit inside a luminescence spectrophotometer, and a syringe pump was used to flow a bath solution containing glucose or no glucose into the flow cell unit for validating the response of the sensor to glucose concentrations. The FRET signals generated from the protein in response to changes in glucose concentrations in the flow-through solution were continuously recorded and used as the basis of CGM.

First, we determined whether the hollow fiber membrane provides a good optical property that allows for effective detection of the sensor's FRET signals in response to glucose concentrations. We mounted the aforementioned AcGFP1-GBPcys-mCherry sensor on a luminescence spectrophotometer and scanned the fluorescence emission spectra of the sensor protein in the absence of glucose. The fluorescence emission spectra obtained from the sensor assembly was compared with those obtained by performing the measurement in quartz cuvette. As shown in Fig. 3, the sensor protein exhibited two characteristic emission peaks at 505 nm (AcGFP1) and 610 nm (mCherry) when excited at 475 nm in the absence of glucose, similar to those observed when the protein was placed in a quartz cuvette. This experiment clearly suggested that the hollow fiber membrane selected for sensor fabrication does not interfere with the FRET measurement. It also indicated that the amount of sensor proteins enclosed inside the hollow fiber membrane is sufficient for inducing a measurable FRET signal. Furthermore, this experiment confirmed that the configuration of the sensor system works well for inducing FRET signals caused by resonance energy transfer from the donor AcGFP1 to the acceptor mCherry. Although the sensitized fluorescence emitted from mCherry is relatively small, the well separation of the excitation spectrum of mCherry from the emission spectrum of the AcGFP1 could significantly reduce background noise from crosstalk or bleed-through, improving the reliability of the sensor. As shown in other studies, the sensitivity and reliability of a FRET protein sensor depend upon the distance and orientation between a donor and its acceptor fluorescent protein as well as the overlap between the excitation and emission spectra of the donor and acceptor proteins (Piston and Kremers 2007; Roy et al. 2008). In this case, the distance and orientation of the AcGFP1 and mCherry protein are determined by the molecular structure of both GBP and the AcGFP1/mCherry FRET pair proteins. In other words, the molecular design of the AcGFP1-GBPcys-mCherry determines the distance and orientation between AcGFP1 and mCherry. The linker between GBP and AcGFP1/mCherry was optimized to achieve maximum FRET efficiency, as described previously (Ye and Schultz 2003). However, crosstalk or spectral bleed-through can be prevented remarkably by carefully selecting a pair of FRET fluorescent proteins. In the past, ECFP and EYFP have been extensively used for constructing FRET molecular probes for detecting intracellular events (Kleemola et al. 2007; Wang et al. 2006). However, a large overlap between the excitation spectra of ECFP and EYFP leads to severe crosstalk or bleed-through between ECFP and EYFP, reducing the reliability of quantitative analysis based on these FRET detections (Goedhart et al. 2007; Tramier et al. 2006). In this study, we showed that AcGFP1 can be paired with mCherry as a FRET pair for quantitative biological analysis. To the best of our knowledge, this could be the first report showing that AcGFP1

could be paired with mCherry for developing a FRET molecular probe or a FRET sensor protein for continuous monitoring of biomolecules.

3.2.2. Using the AcGFP1-GBPcys-mCherry biosensor for CGM—Next, we examined the ability of the AcGFP1-GBPcys-mCherry sensor for CGM (Fig. 4). The experiment was performed by passing a solution containing glucose at various concentrations from 25 μ M to 1.6 mM continuously through the flow cell unit where the AcGFP1-GBPcys-mCherry sensor was installed. A sugar-free buffer was used to flush out glucose from the sensor before introducing another concentration of glucose into the flow cell unit. The FRET signal obtained in the absence of glucose served as a baseline for the sensor. The protein was excited at 475 nm and the FRET signals were recorded continuously every 6 seconds. We observed a gradual increase of FRET signals in response to an increase in glucose concentration in the flow cell unit due to the flow of glucose into the unit. The FRET signal became stabilized at an elevated value when the buffer inside the flow cell unit was completely replaced with the glucose containing bath solution (Fig. 4a). The trend was reversed when the bath buffer containing no glucose passed through the sensor. This observation is in line with early studies where the binding of glucose to the GBP caused the donor fluorescent protein fused at the N-terminus of GBP to drift away from its acceptor fused at the C-terminus of GBP, thereby increasing the donor emission and decreasing the sensitized acceptor emission (Fehr et al. 2003; Ye and Schultz 2003). The FRET signals were clearly correlated to the glucose concentrations. We observed the increase of FRET signals with the increase of glucose concentration in the bath solution passed through the flow cell unit. A glucose response curve of the sensor was made by fitting the Δ Ratio determined from the FRET measurement to the glucose concentration. The Δ Ratio is defined as the difference between the baseline and the maximum FRET signal obtained at certain glucose concentrations. Clearly, there is a very good correlation between FRET signals of the sensor and glucose concentrations in the bath solution (Fig. 4b), suggesting the ability of the sensor for CGM. The response of the sensor to the glucose was very reproducible and stable. We didn't observe any significant shift of the baseline during 2 h of the measurement.

Repeatability of the sensor measurements has also been investigated. As shown in Fig. 4a, glucose concentrations of 50, 100, 200, 400, 800, and 1,600 μ M were spiked through the sensor assembly, followed by 800, 400, 200, 100, 50, and 25 μ M. The repeatability of the sensor response can be easily observed through the symmetry of the response curve (comparable peak heights corresponding to the concentration of glucose that was added in FRET ratio vs. time for various glucose concentration curve). Furthermore, we determined the reproducibility of the sensor fabrication. We fabricated multiple sensors and determined their glucose response curves. Our tests showed a good reproducibility of sensor fabrication. We observed no significant deviation of glucose response curves among these sensors (Supplementary data, Figure S-1).

3.2.3. Interference of other sugars on AcGFP1-GBPcys-mCherry sensor response—The actual sample for glucose detection can contain other sugars that could potentially interfere with the glucose detection by the AcGFP1-GBPcys-mCherry sensor. These interferences should be determined and well characterized. Sugars that we selected for these tests are galactose, lactose, fructose, ribose, mannose, and mannitol. In light of low concentrations of these sugars or sugar alcohols in the blood or body fluids (Arthur et al. 1991; Hui et al. 2009; Ning and Segal 2000; Pitknen 1996), their interference on GBP's glucose-binding activity needs to be determined, as they have a molecular structure very similar to that of glucose. Table 1 summarized the testing results. The sugar concentrations selected for these tests were based on their physiologically significant values reported in literature. For example, serum fructose concentration in patients with diabetes was reported to be around 12.0 ± 3.8 μ M (Kawasaki et al. 2002). Two sets of experiments were performed in

order to ascertain the inference of these sugars on the glucose detection of the sensor. First, we dissolved these individual sugars in the bath buffer to a final concentration of 2 mM and used these bath solutions to determine the inference of these sugars on sensor's glucose detection. While concentrations of these sugars in blood and body fluids are usually well below 2 mM, we decided to examine them at 2 mM. We wanted to know whether the sensor would respond to these compounds when their concentration is close to the saturation concentration of glucose for the AcGFP1-GBPcys-mCherry sensor. The response of the sensor to 1.6 mM of glucose was set as a standard against the response of the sensor to these sugars. As shown in Table 1, the sensor responded to galactose but not to fructose, lactose, mannose, and mannitol. The response of the sensor to the galactose was expected, as the GBP has a high affinity for both galactose and glucose, as revealed previously (Vyas et al. 1991). However, the interference from galactose may be negligible because its concentration in blood and body fluids is usually low (Cong and Stanton 2000). When its concentration was below 75 μ M, its inference on the glucose detection of the sensor could not be detected, as shown in the Table 1. The concentrations of galactose in blood and body fluids are usually below 75 μ M (Cong and Stanton 2000). In the second experiment, we mixed these sugars with glucose and investigated their inference with the sensor's glucose detection. To find out whether the interference of these sugars on glucose detection is significant when their concentrations are close to their physiological range, we included 75 μ M of each sugar with 1.6 mM of glucose containing buffer, and each of these mixtures were passed through the sensor assembly. The glucose measurements obtained from these mixtures were compared with the measurements made from glucose alone. We did not observe any interference in the sensor responses from the compounds tested.

Although here we only investigated the interference of these compounds at two selected concentrations instead of at a range of concentrations, previous studies have suggested that the mutation of GBP at the 16th position of its amino acid residues does not lead to considerable changes in its specificity for glucose (Fehr et al. 2003). Our study here further confirmed these observations. Our experimental data suggested that the interference of sugars relevant to glucose in blood and body fluid is negligible when their concentrations are close to micromolar level.

It is noteworthy that the interference test should be performed using real body fluid samples so one can have better ideas on how other sugars will potentially interfere with the glucose measurement when the sensor is applied for glucose detection in body fluids. However, the prototype sensor developed in this work requires a large sample volume, making such a test impossible at this stage of work. In the next phase, we will miniaturize the sensor assembly and test it in animals such as rats. Upon the availability of the miniaturized sensor, we will perform the interference test using real body fluids. However, the data shown here suggest that interference of other sugars to glucose measurement using this sensor is negligible.

3.2.4. Stability and shelf-life of the sensor—Next we investigated the stability and shelf-life of the sensor after they were fabricated. Ideally, the sensors should be able to be stored at room temperature and have a long shelf-life. For these reasons, we determined the shelf-life of the AcGFP1-GBPcys-mCherrysensor at room temperature. The sensor was stored in 1.6 mM glucose containing buffer during storage. It was assembled in the flow cell unit and tested for CGM at day 2, day 7, and day 21. Fig. 5 shows the glucose response curves of the sensor to glucose ascertained at different days. The FRET measurements were normalized by dividing the sensor signal (Δ FRET Ratio, calculated as the difference between the peak height and the baseline from the CGM response curve at each glucose concentration) with the signal at a saturated glucose concentration of 1,600 μ M. Clearly, the sensor was quite stable up to three weeks when stored at room temperature. Because of the

time constraint, we did not continue testing the shelf-life of the sensor. Thus, the actual shelf-life of the sensor could be longer than three weeks.

3.2.5. Leaching test—As described above, one of the potential applications of the AcGFP1-GBPcys-mCherry sensor protein is the development of an implantable glucose sensor for continuous glucose monitoring in body fluids. The leaching of the sensor protein from the hollow fiber membrane could elicit an immune reaction. Thus it is important to determine whether the AcGFP1-GBPcys-mCherry will be leached out from the hollow fiber membrane before the sensor can be used for *in vivo* CGM. To this end, we investigated the leaching of the AcGFP1-GBPcys-mCherry sensor from the hollow fiber module that we used to enclose the sensor protein. We stored the AcGFP1-GBPcys-mCherry sensor in the binding buffer as described in Materials and Methods at 37°C (our body temperature) for 30 days. The samples were collected from the buffer every day and subjected to emission scan to ascertain the leaching of the sensor protein from the hollow fiber membrane. We did not detect any such leaching (Supplementary data, Figure S-2). As mentioned above, the molecular weight of the sensor protein is about 95 kDa, whereas the molecular weight cut-off of the hollow fiber membrane is about 18 kDa. Thus, we speculated that the sensor protein can be completely enclosed inside the membrane without significant leaching, suggesting the possibility of safe use of the sensor for *in vivo* CGM.

Taken together, the above experimental results evidently demonstrated that the new sensor developed in this study is able to measure glucose concentrations at the micromole range with considerable stability and specificity.

4. Conclusion

In this work, we constructed a novel glucose sensor protein, AcGFP1-GBPcys-mCherry, which responds to glucose in a micromole range. We further demonstrated the fabrication of a glucose sensor using the protein. There are multifaceted advantages with this new sensor protein. First, the sensor developed with this protein can be used for CGM with a high sensitivity in the micromolar range from 25 μ M to 800 μ M. Because of its simple structure, its miniaturization can be easily achieved. A miniaturized sensor assembly may prove ideal for the development of an implantable glucose sensor for CGM in body fluids. The reagentless sensing mechanism described here provides a unique addition to currently available approaches for noninvasive glucose detection. Furthermore, the sensor protein developed in this work has the potential for developing glucose sensors for lab-on-chips devices. In addition, it can possibly be used in living cells for visualizing intracellular glucose dynamics, providing a tool for probing the complex biochemical pathways associated with glucose metabolism. The ability of the quantitative determination of intracellular glucose concentrations may pave a way for developing high throughput screening technologies, where the glucose dynamics of cells under different conditions can be determined in real-time and at a single cell level. The technology developed in this work can be further extended to whole animal imaging for profiling glucose concentrations or changes of glucose concentrations in different tissues in response to various diabetes treatments or drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Glucose binding isotherm of the AcGFP1-GBPcys-mCherry sensor protein

The FRET intensity ratios (AcGFP1/mCherry) at various glucose concentrations were fitted into a one-site saturation binding kinetic curve using Prism5 software to determine the apparent dissociation constant, K_d . Saturation, S , is defined as $(R_{\max} - R)/(R_{\max} - R_{\min}) = n[S]/(K_d + [S])$, with $R_{\max}$ for maximum intensity ratio of the AcGFP1/mCherry at the saturation with glucose, $R_{\min}$ for minimum ratio in the absence of glucose, 'n' the number of binding sites and $[S]$ the glucose concentration. Three independent titrations were performed to determine the K_d value.

Figure 2. A molecular structure of FRET sensor proteins designed for CGM

In this design, a glucose binding protein (GBP) is sandwiched with AcGFP1 at its N-terminus and mCherry at its C-terminus. The GBP adopts an "open" form in the presence of glucose and a "close" form in the absence of glucose, leading to a change in the relative distance between AcGFP1 and mCherry and thereby altering the FRET efficiency between AcGFP1 and mCherry.

Figure 3. Comparison of the fluorescence emission spectra of the AcGFP1-GBPcys-mCherry sensor protein in sensor assembly and quartz cuvette

The sensor protein was excited at 475 nm and its emission spectrum was scanned from 490 to 690 nm. The slit widths were set at 10 nm for both excitation and emission. The measurement was performed at the room temperature in the absence of glucose.

Figure 4. The use of the AcGFP1-GBPcys-mCherry sensor for CGM

(a) The response of the AcGFP1-GBPcys-mCherry sensor to various glucose concentrations. The sensor was stored in glucose (1.6 mM) containing buffer at room temperature until use. Buffers containing various concentrations of glucose were flown through the flow cell unit and the FRET signals were recorded continuously every six seconds. Excitation at 475 nm, Emission at 505 and 610 nm, Slit widths were set at 10 nm for both excitation and emission. Red arrows indicate flow of glucose containing buffer through the flow cell unit, while the green arrows indicate the flow of no glucose containing buffer through the flow cell unit. (b) A glucose response curve of the sensor made from the data shown in the (a). The ΔRatio reflects the difference between the peak height at a given glucose concentration and the baseline. The measurement was performed at room temperature.

Figure 5. Stability and shelf-life of the sensor

The sensor was stored at 1.6 mM glucose containing buffer and stored at room temperature up to three weeks after it was fabricated. The CGM was performed at day 2, 7, and 21. The measurements were normalized by dividing the FRET signal (Δ FRET Ratio, calculated as the difference between the peak height and the baseline from the CGM response curve at each glucose concentration) with the signal for saturating glucose concentration, 1600 μM .

Table 1

Interference of various compound on glucose detection using the sensor

Sugar	Concentration (mM)	Interference	Concentration* (μ M)	Interference
Fructose	2	–	75	–
Galactose	2	+	75	–
Lactose	2	–	75	–
Mannose	2	–	75	–
Mannitol	2	–	75	–

* The sugars were mixed with 1.6 mM glucose in the bath buffer for testing their interference on the glucose detection of the sensor.

“–”, no inference; “+”, have interference.