pH-Insensitive Glucose Indicators

Jared R. Garrett and Xinxin Wu
Biomedical Engineering Program, College of Engineering, University of Arkansas, Fayetteville, AR 72701

Sha Jin
DNA Resource Center, University of Arkansas, Fayetteville, AR 72701

Kaiming Ye
Biomedical Engineering Program, College of Engineering, University of Arkansas, Fayetteville, AR 72701

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There is an urgent need for developing a biosensor that can real-time and noninvasively determine glucose concentration within living cells. In our previous study, we have engineered a glucose indicator protein (GIP) that can provide continuous glucose monitoring through a conformation change-induced Förster resonance-energy transfer measurement. Because of the pH-sensitivity of the fluorescent proteins used in the GIP construction, the GIP made from these fluorescent proteins is less tolerant to a pH change, especially to the acidic environment. It has been well documented that intracellular pH does not always remain the same, and it fluctuates in metabolism and other cellular activities and also differs between cellular compartments. To address these issues, we developed a GIP that can tolerate to pH change. This GIP was constructed by flanking a glucose binding protein with a cyan fluorescent protein and a pH-insensitive yellow fluorescent protein. Our experimental results indicated that the new GIP is more tolerant to pH change. The glucose response of this new GIP kept almost unchanged from pH 7.3 to 5.3, suggesting its capability of tolerating to acidic environment. This capability is desirable for intracellular glucose measurement.
Keywords: glucose indicator protein, intracellular glucose measurement, fluorescence resonance energy transfer, green fluorescence protein, biosensor

Introduction

While various biosensors have been developed to measure extracellular glucose concentration, the continuous monitoring of intracellular glucose has still been a difficult challenge because of the lack of a methodology that is nondestructive to the cells. Currently, the only method to measure glucose uptake is to use a NMR spectroscopy[1,2] or to infer the uptake from complex glucose clamp and tracer infusion techniques.[3] Thus, there is an urgent need for developing a biosensor that can real-time and noninvasively determine glucose concentrations within living cells. The continuous monitoring of intracellular glucose will allow us to determine glucose uptake rates in various cells such as skeletal muscle cells that consume 80% of our body glucose.[4] The accumulation of these data will offer new insights into two critical steps of cellular glucose metabolism: glucose transport and phosphorylation, providing valuable information for understanding the development of type 2 diabetes and obesity in insulin-resistant patients.

To address this issue, we have engineered a glucose indicator protein (GIP) previously.[5] GIP was constructed based on a glucose binding protein (GBP) isolated from *E. coli*.[6] To integrate a signal transduction function into this protein for sensing glucose, we genetically fused two fluorescent proteins to each end of the GBP: i.e., a green fluorescent protein (GFP) at the N-terminus of GBP and a yellow fluorescent protein (YFP) at C-terminus of the GBP, in such a manner that the spatial separation between two fluorescent moieties changes upon glucose binding, thus generating a signal for glucose detection through a conformational change-induced Förster resonance-energy transfer (FRET). Our studies showed that the change in FRET efficiency between GFP and YFP is correlated to the glucose binding because of the rearrangement of the flap region located on the side of the hinge β-sheet of GBP in the presence of glucose, altering the relative position of GFP and YFP fused to each end of the GBP. Our experimental results further demonstrated that GIP responds well to glucose in a range of 0–20 μM.

However, the mean intracellular glucose concentration is around 100 μM in normal subjects and 200 μM in patients with diabetes.[1] To improve the glucose response range of GBP, Fehr et al. have constructed a variety of GBP mutants and demonstrated that the dissociation constant (K_d) of GBP can be manipulated from 5 to 589 μM through site-directed mutagenesis.[7] Using the mutated GBP (GBP_m), they

Correspondence concerning this article should be addressed to K. Ye at kye@uark.edu.

constructed a GIP by flanking the GBP with a cyan fluorescent protein (CFP) and a YFP at its two ends and demonstrated that the engineered GIP can be used to determine the glucose uptake in COS 7 cells through the intensity FRET microscopy imaging measurement.[7]

Nevertheless, it is evident that these GIPs and their respective glucose measurements are influenced by the environmental pH in which they operate. For example, both CFP and YFP are relatively acid-sensitive and they can be quenched by chloride ion.[8–13] It was found that the pK_a of EYFP is only about 6.9.[11] The influence of pH on YFP can be problematic in microscopy imaging of YFP within living cells, as intracellular pH does not always remain the same and it fluctuates in metabolism and other cellular activities[14–16] and also differs between cellular compartments.[10] For example, in HeLa cells, the pH in endoplasmic reticulum is about 7.2 ± 0.2, while Golgi pH is about 6.4 ± 0.3.[17] Further studies reveal that the intracellular pH can vary from 0.1 to 1.6 unit during metabolic and developmental transitions in a variety of cells.[14,18] It is also found that the glucose-induced insulin release can cause a significant decrease in the intracellular pH.[19] Thus, it is highly desired to develop a pH-insensitive GIP for continuous glucose monitoring through in vivo fluorescence microscopy measurement.

In this study, we used a pH-insensitive YFP[11] to design and construct a pH-insensitive GIP for continuous glucose monitoring. The goal of this study is to determine whether the pH tolerance of GIP can be improved by coupling a pH-insensitive YFP (YFP_i) with an ECFP in a conformation-induced FRET measurement.

Materials and Methods

Fusion protein construction

To construct a pH-insensitive glucose indicator, referred as C_oY_i, we first subcloned enhanced CFP (ECFP) into the vector pTAGBP[5] through Nco I and Kpn I enzyme sites directly at the downstream of the *trc* promoter, followed by GBP_m[7] at Kpn I and Spe I sites and YFP_i at Hind III and Spe I sites. The GBP_m and YFP_i were constructed through site-directed mutagenesis, as suggested elsewhere.[7,11] The ECFP was amplified from pECFP (Clontech, CA). The 5′ and 3′ primers used were 5′-TCATTCCCATGGTGAGCAAGGGCGA-3′ and 5′-TCATTCAAGCTTTCATTCACTAGTCCACCGGTACCGGCGCCT-3′, respectively. The YFPi was amplified from pYFPs.[11] The 5′ and 3′ primers for YFP_i insertion were 5′-TCATTCACTAGTGGTGAGCAAGGGCGAGGAGC-3′ and 5′-TCATTCAAGCTTGCTTGTACAGCTCGTCCATGC-3′, respectively. These pair of primers was also employed to amplify the EYFP from pEYFP (Clontech). Extra base pairs were introduced in the fusion gene to adjust the insertion into a proper codon reading frame. The linker between ECFP and GBP_m, GBP_m and YFP_i were optimized, as described previously.[5] Similarly, a pH-sensitive GIP, referred as C_oY_o was constructed by flanking the GBP_m with ECFP and EYFP.

E. coli DH5α (F⁻ Φ80c*lac*ZΔM15 Δ(*lac*ZYZ-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (rk⁻mk+) *deo*R *thi- pho*A *sup*E44 λ *gyr*A96 *rel*A1) was used for plasmid proliferation and protein expression. Luria-Bertani (LB) media supplemented with 100 μg/mL of ampicillin were employed to select positive colonies on the LB plates. All the restriction enzymes were purchased from New England Biolabs, and the high fidelity PCR polymerase, *Pfu*, from Stratagene was used in all PCR reactions. A quick ligation kit from Roche was applied for fast vector ligation at room temperature. All evolved chemicals were of molecular biology grade, purchased from Sigma.

All the sequences of positively selected plasmids were verified through DNA sequencing.

Protein purification

A portion of 2.5 μL of recombinant *E. coli* DH5α was inoculated into 5 mL of LB medium supplemented with ampicillin (100 μg/mL) and glucose (2 g/L). After overnight incubation at 37°C with shaking at 225 rpm, a portion of 2 mL of cultures was transferred to a flask containing 100 mL of LB broth containing ampicillin (100 μg/mL) and glucose (2 g/L). Glucose was added because it represses the basal level transcription from the *trc* promoter, thus stabilizing the gene that was inserted downstream of the *trc* promoter. This culture was incubated at 225 rpm at 37°C until OD_{600} became 0.5–0.7. Isopropyl β-D-1-thiogalactopyranoside (1 mM; Gold Biotechnology, MO) was then added to the medium to induce the expression of the GIPs. Cell pellets were collected 4–5 h postinduction through centrifugation at 4,000 rpm for 15 min at 4°C. The pellets were stored −20°C until use. To extract the GIPs from *E. coli*, we used the B-PER II protein extraction kit from Pierce. In brief, the frozen cells were resuspended in 6.5 mL of the B-PERII reagent supplemented with 1 mM of phenylmethanesulfonyl fluoride (PMSF) to prevent degradation of the proteins. PMSF was freshly made in dimethyl sulfoxide. After incubation for 20 min at room temperature with gentle shaking, the supernatants were collected through centrifugation at 15,000 rpm for 15 min at 4°C. The collected supernatants were kept at −20°C until use.

To purify the GIPs using immobilized metal ion chromatography (IMAC), we fused a $(His)_6$ tag to the C-terminus of the GIPs, as described previously.[5] A HiTrap (GE Healthcare Lifescience, NJ) IMAC column was used to purify the fusion proteins. The column was assembled and washed with 15 mL of distilled water at a flow rate of 1 mL/min. Then, a portion of 2.5 mL 0.1 M $NiSO_4$ was loaded into the column at a flow rate of 1 mL/min. The column was then equilibrated with 30 mL of wash buffer (20 mM Tris-HCl, 500 mM NaCl, and 20 mM Imidazole) at a flow rate of 4 mL/min. The washing buffer was adjusted to a pH of 7.5 using 0.1 M HCl. To prevent from clotting inside the column, the cell-free extracts were filtered through a 0.45-μm low-protein binding membrane filter. The filtered proteins were then applied to the column at a flow rate of 1 mL/min. The column was subsequently washed with 30 mL of wash buffer at 4 mL/min. As noted previously, this wash buffer contains 20 mM imidazole to reduce nonspecific binding. The proteins were then eluted with 30 mL of elution buffer (20 mM Tris-HCl, 500 mM NaCl, and 500 mM imidazole) at a flow rate of 1 mL/min. The elution buffer was adjusted to a pH of 7.5 using 0.1 M HCl. The eluent was collected into Eppendorf tubes using a fraction collector (Bio-Rad). Each fraction's OD_{280} was measured with a spectrophotometer. The fraction with the highest OD_{280} absorbance was collected and used for the glucose binding assay.

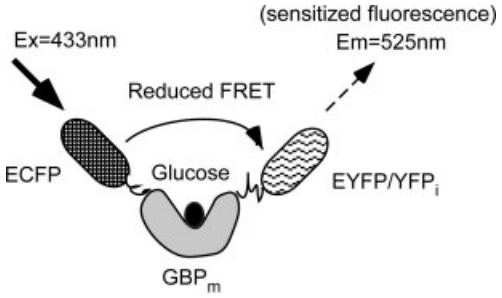

Figure 1. A schematic diagram of the GIP for continuous glucose monitoring.

The GIP was constructed by flanking one mutated glucose binding protein (GBP_m) with two fluorescent proteins. The GBP_m changes its conformation upon the glucose binding, leading to a change in the distance between ECFP and EYFP or a pH-insensitive YFP_i. This glucose binding-induced distance change alters the Förster resonance-energy transfer efficiency that can be quantitatively measured by detecting the change in the intensity ratio of fluorescence emitted from both EYFP and ECFP, as described in the Materials and Methods section.

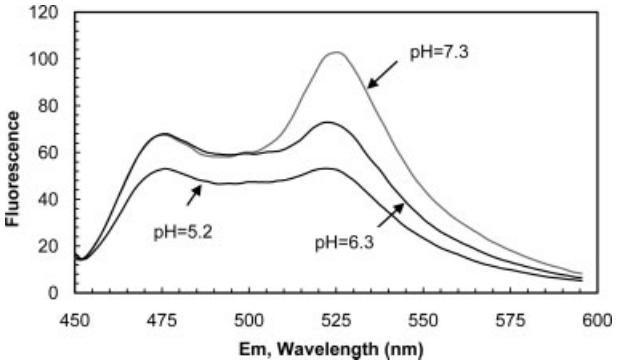

Figure 2. The effect of pH on the glucose response of C_oY_o.

The proteins were excited at 433 nm and the fluorescence spectra of both ECFP and EYFP were scanned with a slit width of 10 nm. The protein was exposed to 0.4 mM of glucose at room temperature under different pH.

Dialysis

To remove the imidazole from the purified proteins, we dialyzed the proteins against sugar-free dialysis buffer (20 mM Tris-HCl, 5 mM dithioerythritol (Sigma, WI), 150 mM NaCl, and 1 mM $CaCl_2$) at 4°C. The proteins were placed in Float-A-Lyzer Dialysis Tubing (Fisher Scientific, PA) with a 25-kDa molecular weight cutoff of and dialyzed against ~600 mL of dialysis buffer. The dialysis buffer was exchanged with fresh dialysis buffer after 2 h. After another 2 h, the dialysis buffer was exchanged again and left overnight. After dialysis, the proteins were removed from the dialysis tubing and placed into an Eppendorf tube. The protein concentration was measured spectrophotometrically at 595 nm using Coomasie Bradford Protein Assay Kit (Pierce, IL). The purity of the proteins was analyzed through SDS-PAGE assay, as described previously.[5]

FRET measurement

The response of the fusion proteins, C_oY_o and C_oY_i, to glucose under different pH values were determined by FRET analysis. In brief, 600 μL of GIPs were transferred to a cuvette and placed in a luminescence spectrophotometer (Perkin-Elmer LS 55). The fluorescence intensities of ECFP and YFP_i or EYFP were monitored at 476 and 526 nm, respectively, when excited at 433 nm. The intensity ratio of YFP_i over ECFP was calculated and correlated to the glucose concentrations to establish a calibration curve under various pH values. The pH of the GIP solution was adjusted to different values using 0.1 M HCl, and the highly concentrated glucose solution was employed to titer the glucose response curve of the GIPs under different pH values. To reduce the volume effect on the fluorescence measurement, the total volume of glucose added to the GIP solution during the entire titration was less than 12 μL. The slit width for both excitation and emission wavelengths was set at 10 nm. The fluorescence intensities of the EFCP and YFP_i were measured 10 min after the addition of glucose to the GIPs solution.

Results

The effect of pH on the response of GIP to glucose

In this first experiment, we determined the effect of pH on the response of GIP to glucose. Using the same strategy as described in our previous report,[5] we constructed a C_oY_o in which a GBP_m[7] was flanked with a ECFP at its N-terminus and a EYFP at its C-terminus, as shown in Figure 1. The fusion proteins were expressed and extracted from *E. coli*. After purification and dialysis against a sugar-free binding buffer, a portion of 600 μL of the protein was used for glucose titration. The pH of the protein solution was adjusted to various values from 7.3 to 5.2 in order to investigate the effect of pH on GIP's response to glucose. Highly concentrated glucose was added to the protein solution, and the fluorescence ratio measurements were conducted with a Perkin-Elmer's luminescence spectrophotometer by exciting the protein at 433 nm and detecting the cyan fluorescence emitted from ECFP at 476 nm and sensitized yellow fluorescence from EYFP at 525 nm. The spectra of the C_oY_o under different pH values were presented in Figure 2. The experiments were performed in triplicate and only a typical spectrum recorded from the C_oY_o when it was exposed to 0.4 mM of glucose was presented in this figure. From these data, you can see that the pH effect on the glucose response of GIP was mainly caused by the pH sensitivity of EYFP. The fluorescence intensity of EYFP reduced to about 30%, while the fluorescence intensity of ECFP remained almost the same when pH dropped from 7.3 to 6.2. The fluorescence of ECFP was slightly affected by pH when it was below 5.2. We observed about 22% reduction in ECFP fluorescence and 48% decrease in EYFP fluorescence when the pH dropped below 5.2. The fluorescence intensity ratios of EYFP to ECFP, which indicated that the glucose binding induced FRET between ECFP and EYFP, were 1.52 at pH 7.3, 1.05 at pH 6.2, and 0.99 at pH 5.2, respectively. This experiment clearly demonstrated that pH has a significant effect on the intensity ratio FRET measurement of GIP when the pH varies from 7.3 to 6.2.

Construction of a pH-insensitive GIP

To alleviate the pH effect on the conformational change-induced FRET upon the binding of glucose to GIP, we constructed a pH-insensitive GIP by flanking GBP_m with a ECFP and a pH-insensitive YFP_i at its N and C termini, respectively (Figure 1). The GBP_m has a pK_d of 589 μM for glucose and pK_d of 848 μM for galactose.[7] Thus, the GIP constructed with this GBP_m will be able to measure glucose concentration up to 5 mM. The new GIP was referred as C_oY_i. The reason that we did not adopt a pH-insensitive CFP to construct a pH-insensitive GIP is that ECFP is quite stable in the pH ranges that we studied, as described earlier. In addition, it is difficult

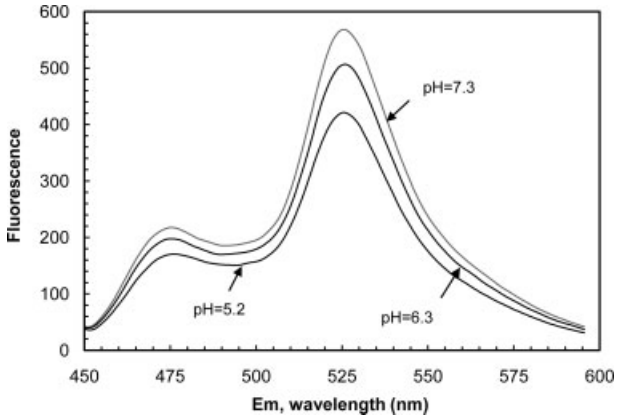

Figure 3. The effect of pH on the glucose response of C_oY_i.

The proteins were excited at 433 nm, and the fluorescence spectra of both ECFP and YFP_i were scanned with a slit width of 10 nm. The proteins were exposed to 0.4 mM of glucose at room temperature under different pH.

to find an appropriate pH-insensitive CFP to pair with YFP_i for the FRET. YFP_i has a pK_a of 6.0, and has its maximum excitation wavelength at 515 nm and its maximum emission wavelength at 528 nm.[11] Thus, YFP_i is quite stable in an acidic solution. Figure 3 presents the response of C_oY_i to glucose (0.4 mM) under different pH. We determined the fluorescence intensity ratios of YFP_i to ECFP under different pH values. They were 2.63 at pH = 7.3, 2.56 at pH = 6.3, and 2.50 at pH 5.2. This corresponds to a 2.7% of drop in FRET efficiency when pH drops to 6.3 and a 4.9% of drop when pH is below 5.2. Clearly, the glucose binding-induced FRET was not significantly affected by pH for the new GIP protein. These tests suggested that the C_oY_i is a pH-insensitive GIP.

Glucose measurement using a pH-insensitive GIP

Next, we determined the binding isotherm of C_oY_i for glucose. To establish a calibration curve of C_oY_i, we added the highly concentrated glucose directly to the protein solution and measured the change of fluorescence intensity ratios of YFP_i over ECFP. Figure 4 showed the glucose titration results. All of the experiments were carried out at room temperature, and the intensity ratio was measured 10 min after the addition of glucose to ensure the binding of glucose to the GIPs. Our experiment indicated that 10 min was sufficient for glucose binding to the GIPs as the fluorescence intensity ratio tended to be stable after 10-min incubation at room temperature (data not shown). These observations are consistent with our previous studies on the glucose binding to GIPs.[5] As previously reported, the conformational change-induced FRET can be determined by fitting the substrate titration curves to the equation for the binding of a ligand to a protein.[7,20]

$$\frac{R_{max} - R}{R_{max} - R_{min}} = \frac{n[S]^n}{K_d + [S]^n} \quad (1)$$

where [S] is the glucose concentration, K_d is the apparent dissociation constant that corresponds to a glucose concentration yielding an R (fluorescent intensity ratio of YFP to ECFP) midway between R_{max} and R_{min}, n is the Hill coefficient. The $(R_{max} - R)/(R_{max} - R_{min})$ is defined as a saturation of the FRET.

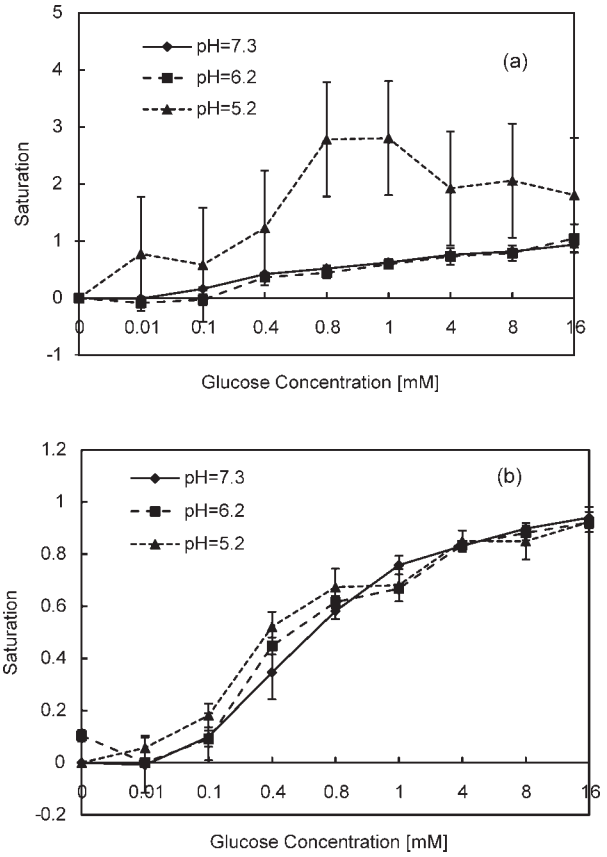

Figure 4. Glucose titration curve for GIPs at varying pH values.

(a) pH-sensitive GIP. (b) pH-insensitive GIP. The experiments were performed at room temperature, and glucose was directly added to the GIP solution to determine the glucose response curve. The slit width for both excitation and emission wavelengths were set at 10 nm. The fluorescent intensities of the ECFP and YFP were measured 10 min after the addition of glucose. The data shown are the mean of three experiments and the error bars show the standard deviations (±SD).

As shown in Figure 4a, the glucose titration curve of C_oY_o at pH = 6.2 was almost the same as that determined at pH = 7.3. Nevertheless, the response of C_oY_o to glucose became fluctuated when pH dropped to 5.2 because of the instability of EYFP under acidic environment. In contrast, C_oY_i exhibited a high stability under an acidic environment. No significant deviation in the glucose titration curves was found when exposing the C_oY_i to an acidic environment. From Figure 4b, we found that the glucose titration curves of C_oY_i obtained at pH = 7.3, 6.2, and 5.3 were almost the same, showing much improvement in reporting a quantitatable and consistent glucose measurement in a varying pH environment. Therefore, this would make an excellent choice of a GIP for intracellular glucose studies where the pH values could vary significantly.

An observation worth noting is the change of the C_oY_o glucose indicator in the dynamic range of the YFP/CFP ratio under acidic environment. The resulting loss of functionality of the YFP in low pH has also been reported by Kim and Cha's group.[9] This group utilized the functionality of FRET occurring between CFP and YFP to measure antimicrobial activity. They showed a significant decrease in FRET from CFP to YFP when the pH is lowered. Lowering of pH resulted in the instability of the YFP molecule causing the

decreased FRET.[8,9,11,12,21] We surmise that this is the mechanism responsible for the loss in the functionality of C_oY_o. Studies published by another group on the measurement of pH in multiple organelles and cytosol using a CFP-YFP protein reported on the sensitivity of YFP to pH as well.[10] It has been speculated that the pH effect on YFP could be due to the increased amount of protons resulting from a lowered pH protonating the polar and uncharged glutamine residue at position 69 of YFP that is in relative close contact to the chromophore of YFP.[22]

By using a pH-stable YFP to construct a glucose indicator C_oY_i, we were able to show that the C_oY_i is more tolerant to the acidic environment. The resulting conclusion from this work is that a pH-insensitive glucose indicating protein can be engineered through flanking a GBP with pH-insensitive fluorescent such as YFP_i used in this work. This protein allows for the quantitation of glucose concentration despite a changing pH environment. The idea and production of a pH-insensitive GIP is a great tool for use in varying pH environments. A perfect application is the measurement of intracellular glucose. The use of this GIP as a noninvasive glucose measurement instrument will work well as interstitial glucose levels tend to follow blood glucose concentrations within a 3- to 5-min time frame.[23] This will further the science of the cell, most notably glucose metabolism and further the research of diabetes.

Another issue which can make the in vivo application of C_oY_i problematic is the specificity of the GBP_m to the glucose. Other sugars that appear inside the cells can compete for glucose binding sites of the GBP_m and thus cause the offsetting of the glucose measurement. However, Fehr et al. have studied the specificity of the GBP_m and concluded that it has a very high specificity to both glucose and galactose[7] because of its unique geometry structure.[24,25] In light of the possibility of the galactose measurement by C_oY_i, this effect can be negligible since the concentration of galactose is usually very low inside cells.

Acknowledgments

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