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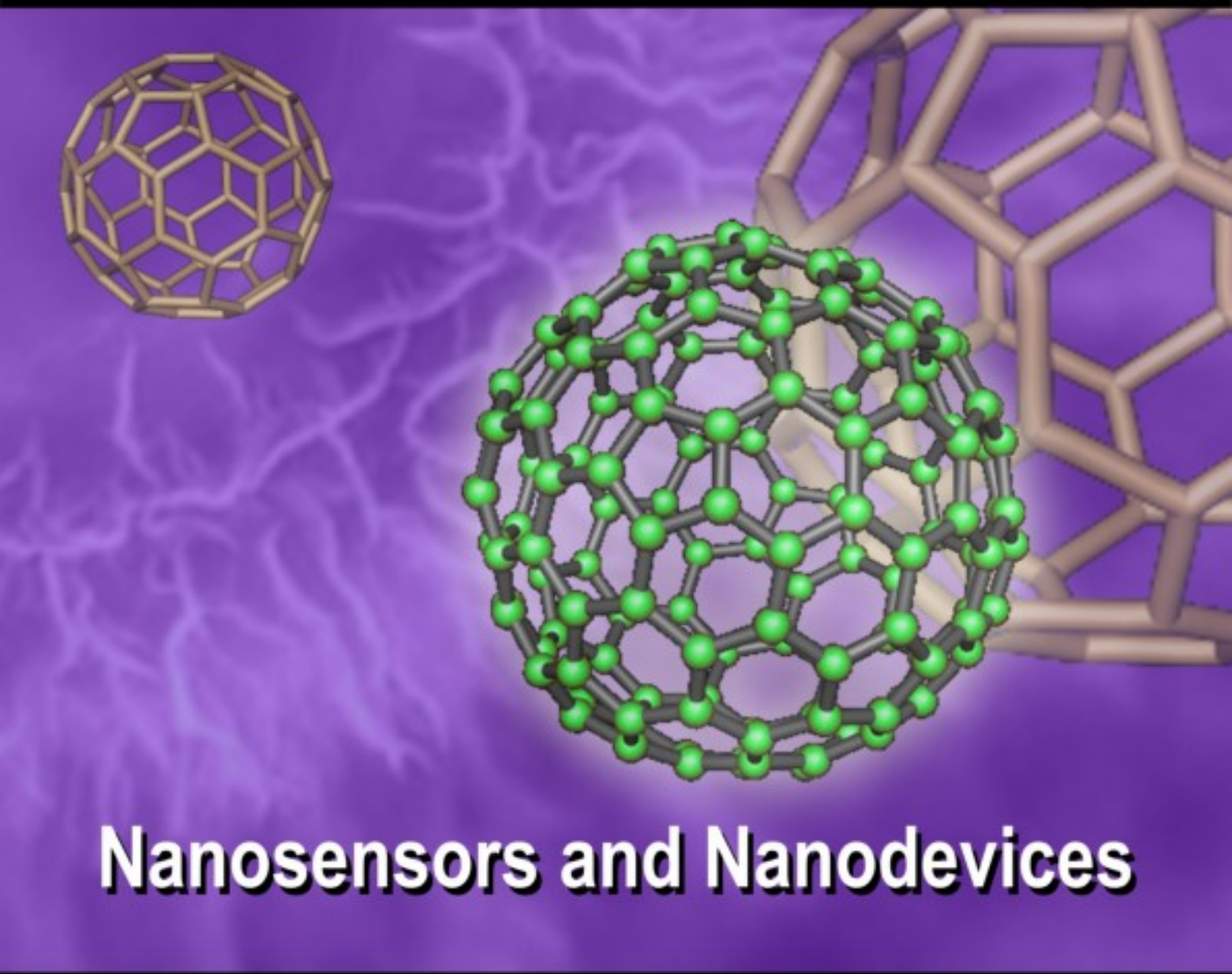

## Nanosensors and Nanodevices

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# Glucose Binding Protein as a Novel Optical Glucose Nanobiosensor

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**Abstract:** Development of an *in vivo* optical sensor requires the utilization of Near Infra Red (NIR) fluorophores due to their ability to operate within the biological tissue window. Alexa Fluor 750 (AF750) and Alexa Fluor 680 (AF680) were examined as potential NIR fluorophores for an *in vivo* fluorescence resonance energy transfer (FRET) glucose biosensor. AF680 and AF750 found to be a FRET pair and percent energy transfer was calculated. Next, the tested dye pair was utilized in a competitive binding assay in order to detect glucose. Concanavalin A (Con A) and dextran have binding affinity, but in the presence of glucose, glucose displaces dextran due to its higher affinity to Con A than dextran. Finally, the percent signal transfer through porcine skin was examined. The results showed with approximately 4.0 mm porcine skin thickness, 1.98 % of the fluorescence was transmitted and captured by the detector. *Copyright © 2009 IFSA.*

**Keywords:** FRET, NIR, Dextran, Concanavalin A, Glucose biosensor

## 1. Introduction

*In vivo* glucose sensing has gained more focus by scientist and researchers, but remained a difficult area due to the number of problems, such as biofouling, poor optical transmission, and poor glucose sensitive probes. There are several enzymes used to detect glucose. But a major problem with these enzymes is the byproduct produced. In the case of glucose oxidase, the hydrogen peroxide will cause major problems with the optical dyes that are pH sensitive. To overcome this problem, Glucose Binding Protein (GBP) will be used to detect glucose. In this research project the GBP structure provides a glucose binding site in a shape of a pocket. When glucose is introduced, it will bind to the glucose binding sites and will cause a conformational change.

The glucose binding protein of *Escherichia coli* (*E. coli*) is considered as an initial component for both chemotaxis towards glucose and high-affinity active transport of sugar. *E. coli* is a bacterial periplasmic binding protein. It is an essential component that serves as initial receptor for active transport systems [1]. The X-ray structures of this protein have provided a molecular view of the sugar-binding site and of the site for interacting with the Trg transmembrane signal transducer [2]. Fig. 1 provides a schematic of the geometry of the sugar-binding site. It is located in the cleft between the two lobes of the bilobate protein. It is designed for tight binding and sequestering of either the alpha or beta anomer of the D-stereoisomer of the 4-epimers galactose and glucose [3]. The diameter of glucose binding protein (GBP) is 50 Å [4].

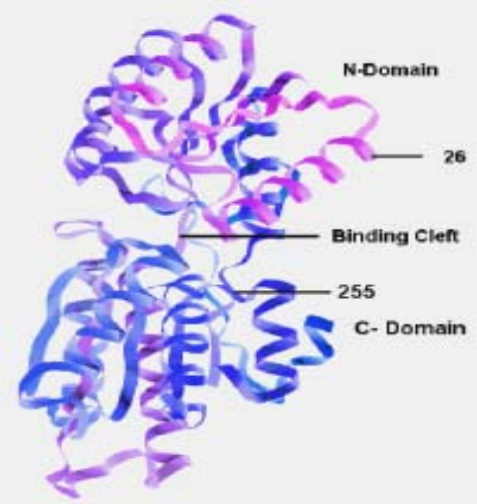


**Fig. 1.** Schematic of the geometry of the glucose-binding site [5] (http://talaga.rutgers.edu/people/images/GBP.png).

Mature GBP consists of 309 amino acids with a molecular weight of 33,310 Da [6]. It is ellipsoidal in shape with two different but similarly folded domains connected by three different peptide segments that serve as a flexible hinge and produce a cleft between the two folded domains. The ligand-binding site is deep within this cleft. Each domain has a core of six β-sheet strands flanked by two or three helices on both sides [7]. In the absence of D-glucose, the two domains remain far apart with the cleft accessible to solvent. Glucose induces a hinge motion in GBP and the sugar becomes completely engulfed in the deep cleft between the two domains. This conformational change in GBP results in the exclusion of solvent molecules from the binding site and enables efficient hydrogen-bonding interactions between the sugar and the residues in the binding site. D-glucose binds to GBP with dissociation constants, *K*d, of 0.4 mM [8]. The conformational change produced in GBP upon binding glucose constitutes the basis of FRET nanobiosensor development strategy.

AF750 and AF680 is a viable FRET pair that will be used to label the GBP. Wild-type *E. coli* GBP has multi sites of amino but no cysteine moieties. By incorporating a unique cysteine to the protein, this will permit site-specific labeling with fluorophore and the optimization of the induced fluorescence change in the presence of glucose.

Site-directed mutagenesis was performed to incorporate a single cysteine in GBP at site 175 to produce GBP mutant. This site was chosen because the crystal structure of GBP [9] shows that these residues are located near the binding cleft. The response of this labeled mutant GBP upon binding with sugar was monitored by following the changes in the fluorescence intensity of the probes. Calibration plots were then constructed by relating the changes in signal with the amount of ligand present.

## 2. Material and Methods

### 2.1. Material

Glucose binding protein from *E. Coli* that contains a unique cysteine at position 175 was purchased from Senseomics (Lexington, KY). Dithiothceitol (DTT) was purchased from Sigma Chemical Co. (St. Louis, MO). β-D-Glucose and PBS also were purchased from Sigma Chemical Co. (St. Louis, MO). Alexa Fluor 750 C5-maleimide, which was used to label the unique cysteine on the GBP, was obtained from Invitrogen. The Alexa Fluor 680 caboxylic acid, succinmidyle ester was also purchased from Invitrogen. DMSO 78.13 MW was obtained from the MU Chemical Recycling (Columbia, MO). Dialysis tubing 6000-8000 MWCO was obtained from Fisher Scientific (Fairlawn, NJ).

### 2.2. Instrumentation

A UV-Vis absorbance spectrometer (Beckman DU 520) was used to collect absorbance spectra. The slit size (4 nm) and scanning speed (595nm/min) were held constant throughout all the experiments. A scanning fluorescence spectrometer (FluoroMax-3 Jobin Yvon, Hobira) was used to collect fluorescence emission spectra by exciting the sample at 680 nm. The slit size and integration time were 7nm and 0.3s, respectively.

### 2.3. GBP Purifying

The GBP was mixed in Tris-HCl buffer. The addition of Dithiothreitol (DTT) to the GBP was carried out to reduce the disulfide bonds in the GBP. This was accomplished by making a 1 M solution of DTT and then adding GBP. Then the solution was dialyzed in PBS to eliminate the excess DTT. With this process the GBP was ready to allow the cysteine site to be conjugated. The labeling of GBP with AF750 was prepared by using the labeling kit protocol from Ivitrogen.

Bio-Rod experiment was conducted to determine the final protein concentration and the degree of labeling. The final GBP-AF750 concentration was 0.8091 mg/ml and the degree of labeling (DOL) was 1 since there was only one cysteine.

### 2.4. AF680 Labeling on the GBP Amine Sites

By following the protein conjugation protocol from Ivitrogen; the AF680 was conjugated on the GBP amino sites. The conjugated stock was dialyzed in PBS to remove the excess AF680. Then the final conjugated GBP concentration was determined by using a Bio-Rod kit. The final concentration was 0.7842 mg/ml. The DOL was calculated according to this formula

$$\mathbf{DOL= (A\ max\ X\ MW) / ([Protein]\ X\ Є\ dye),}$$

where $A_{max}$ is the absorbance at the maximum peak of the fluorophore used in conjugation, and $\varepsilon_{dye}$ is the extinction coefficient at $\lambda_{max}$ in $cm^{-1}M^{-1}$, and [M] is the molarity of the agent. MW is the molecular weight of the protein. The DOL was 1.5, which means that for every mole of protein there was 1.5 moles of dye.

### 2.5. The existence of AF 680 and AF 750 on the GBP

Experiments were conducted in 2 ml total volume of PBS. First, 11.32 ug of GBP-AF750 was placed in the 4 ml cuvette. It was scanned for background signal of the AF750. The excitation was set at 750 nm with slit opening at 5. The increment was set at 1 nm and the integration time was at 0.3 sec. Second, 11.32 ug of GBP-AF680-AF750 was placed in the 4 ml cuvette with 2 ml PBS. The scanning fluorescence spectrometer was adjusted to conduct the experiment according to the fluorophore used. The excitation was set at 680 nm, which was the excitation wavelength for the AF680 located on the amine sites. The increment was set at 1 nm and the integration time was at 0.3 sec.

### 2.6. Baseline scan from the FRET pair labeled GBP

It is imperative to establish baseline scans from the FRET labeled GBP. The baselines provide background spectra prior to the addition of glucose.

### 2.7. Glucose Response Experiments

After 2 hr incubation of the 1.5684 μg of labeled GBP in PBS buffer probe samples were tested for glucose response. The tests consisted of six additions of glucose concentrations, 0 to 60 μM. For each glucose addition, 5 min. was allowed for incubation at room temperature. Calibration plots were obtained by relating the average fluorescence change with the concentration of glucose in the sample. The data points shown in the figures are the average of three measurements ±1 SD.

## 3. Results and Discussion

### 3.1. GBP Labeling and Fluorescence Resonance Energy Transfer (FRET) Experiments

Fig. 2 indicates a successful labeling of the AF750 on the one cysteine site and AF680 on the amino sites. When GBP-AF680 was excited at 680 nm, an emission peak signal was detected at 702 nm. After the labeling of AF750, the labeled GBP with AF680 and AF750 was excited at 680 nm. There were two emission peaks. One peak was at 702 nm, which is an indication of the presence of AF680, and another peak was at 776 nm, which is an indication of the presence AF750.

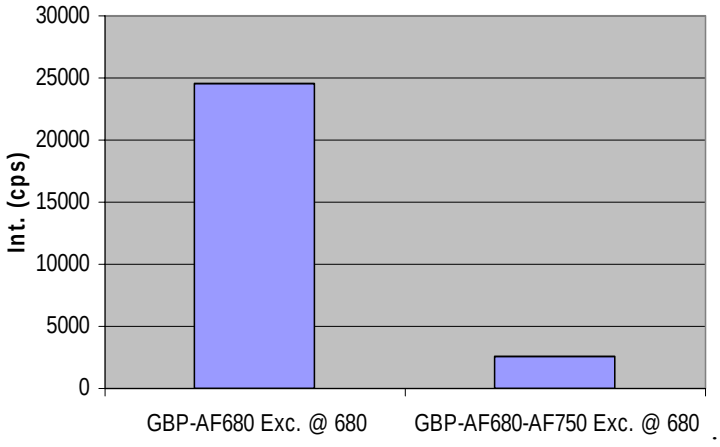


**Fig. 2.** Background signal of the labeled GBP-AF750-AF680.

The successful labeling of GBP with the NIR FRET dye pair will allow us to quantify FRET results after exposure to glucose. When the GBP was first labeled with the donor, AF680, a scan of GBP-AF680 in PBS was obtained. The maximum peak from the AF680 without the presence of the donor was 24480 CPS as shown in Fig. 3. The labeled GBP with AF680 and AF750 was scanned, the donor peak result was 2610. Both peaks of AF680 without the presence of the acceptor and with the presence of the acceptor was used to quantify the energy transfer that took occurred between the donor and the acceptor prior to glucose additions.

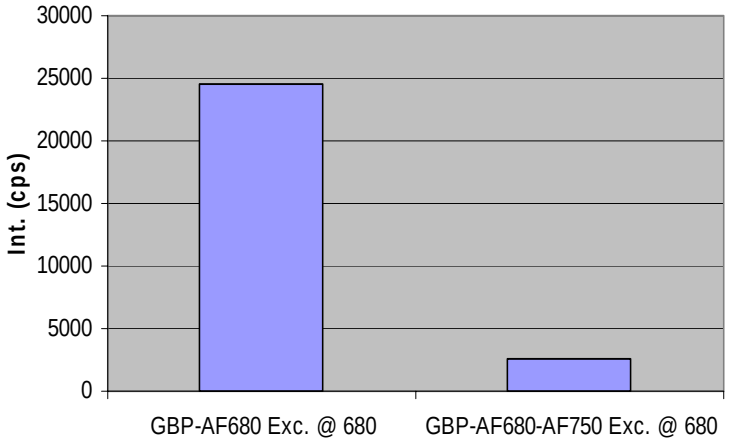


**Fig. 3.** Donor peaks at 702 nm with and without acceptor presence.

The Equation below was used in the quantification.

$$\% \text{ Energy transfer} = 1 - I_{DWA} / I_{D},$$

where $I_D$ is the donor's intensity without acceptor and $I_{DWA}$ is the donor's intensity with acceptor present. The result indicated an energy transfer of 89.34 %. These results provided conclusive observations of energy transfer in terms of an increase in the acceptor peak and decrease in the donor peak prior to glucose additions.

### 3.2. GBP Glucose Response Experiments

Since it was determined that the AF680 and AF750 fluorophores were labeled to the GBP, the next step was to utilize the fluorophores in a FRET-based glucose biosensor. In these experiments glucose response was studied by utilizing1.56 μg of GBP-AF750-AF680 in 2 ml PBS as a baseline and adding various concentrations of glucose.

The labeled GBP was incubated in the PBS for 2 hours in PBS. Then three different D-glucose concentrations were added: 10 uM, 40 uM, and 120 uM. The scanned data was plotted as donor/acceptor (D/A) ratio vs. glucose concentrations. As shown in Fig. 4, there was an exponential decrease to the ratio as glucose concentration was increased. This change in the signal is due to the conformational changes of the GBP. As glucose binds to its GBP binding site, the distance between the labeled sites was changed. The results of the increase in the acceptor peak and decrease of the donor peak indicated a decrease in the distance between the donor and the acceptor, resulting in an increased in energy transfer.

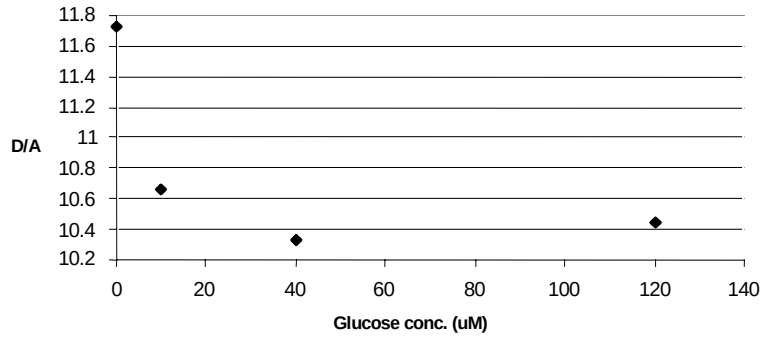


**Fig. 4.** D/A vs. glucose concentration (detection range 0 to 120 uM).

At 120 uM glucose, the D/A ratio was not consistent with the other two D/A ratios. That result was further investigated by looking into the detection range for the labeled GBP.

A smaller dynamic range of the glucose concentration was tested from 0 to 60 uM. Fig. 5 provided a consistent decrease in sensor response as the glucose concentration was increased.

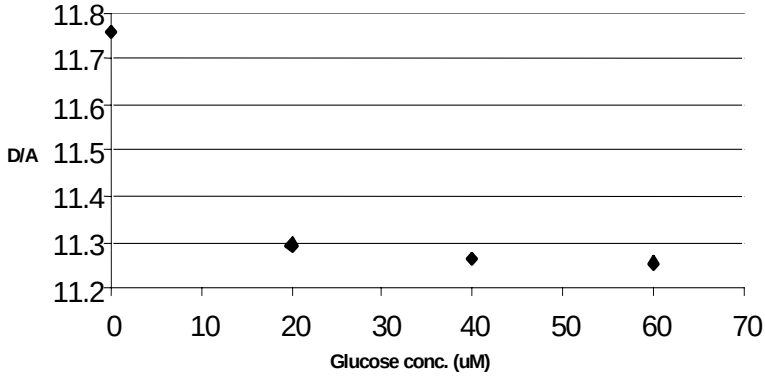


**Fig. 5.** D/A vs. glucose concentration (detection range 0 to 60 uM).

The final experiments were to target improvements in the signal by modifying the labeling process. In the following section, the ratio of mole dye/mole protein was about 3.6:1. We had 8.7 pmole of dye to every 2.38 pmole of protein. There were more glucose concentration additions within the same tested range of 0 to 60 uM.

Glucose response was also studied at t=0 min. and t = 5 min. At t=0 min., the scan of the sample was performed immediately after mixing. No time was allowed for incubation. The result still showed an exponential decrease to the D/A ratio as the glucose concentration increase as shown in Fig. 6.

Fig. 4.6 shows the final graph all three experiments. This result is represented by the following exponential equation was obtained.

$$Y = 10.43\ e^{-0.0006\ x}$$

The $R^2$ value is 0.8583

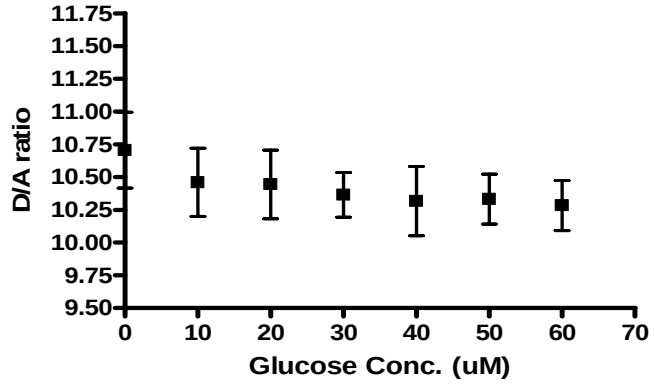


**Fig. 6.** Glucose response to different samples at t=0min.

At t = 5 min., the D/A ratio was more consistent in the exponential decrease as the glucose concentration was increasing as shown in Fig. 7.

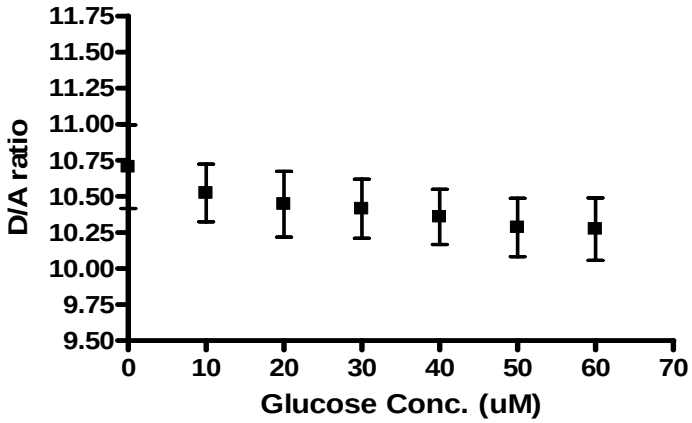


**Fig. 7.** Glucose response of three samples at t=5 min.

Fig. 4.7 shows the final graph all three experiments. By using Fig. 4.7, we got the following exponential equation was obtained.

$$Y = 10.391e^{-0.0005\,x}$$

The $R^2$ value is 0.7317.

It is conclusive that incubation time is very important after the addition of different glucose concentrations.

## 4. Conclusions

Glucose binding protein (GBP) is a monomeric periplasmic protein. It binds glucose with high affinity. The binding mechanism is based on a hinge motion due to the protein conformational change. This change was used as an optical sensing mechanism by applying Fluorescence Resonance Energy Transfer (FRET). With the introduction of a single cysteine at a specific site by site-directed

mutagenesis, a single-label attachment at specific sites with a fluorescent probe, AF750, was ensured. The other sites are amino sites, which were labeled with a donor fluorophore, AF680. Since this residue was not involved in ligand binding and since it was located at the edge of the binding cleft, it experienced a significant change in environment upon binding of glucose. The sensing system strategy was based on the fluorescence changes of the probe as the protein undergoes a structural change on binding. This structure change allowed a reduction in the distance between the donor and the acceptor, which allowed more energy transfer as glucose concentration was increasing. Also by allowing 5 min for glucose to incubate, the respond from the biosensor was decreasing exponentially as glucose concentration was increasing. The detection range was determined between 0-60 uM. The dynamic range could be improved by increasing the GBP concentration.

All the above results can be used in designing more sensitive optical glucose biosensor. These sensors may eventually be deployed for *in vivo* glucose sensing with superior sensitivity and without the concern of toxicity.

## Acknowledgments

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