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Biocompatible Fluorescent Hydrogel Fiber for Glucose Monitoring

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Biocompatible Fluorescent Hydrogel Fiber for Glucose Monitoring

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Abstract:

Diabetes mellitus is one of the leading incurable diseases which may lead to severe health complications due to elevated blood glucose levels. This is mainly caused due to lack of insulin secretion by pancreas (Type I) or when the cells in the body become resistant to insulin (Type II). The key for diabetes treatment is regular monitoring and maintenance of blood glucose levels in the body. We present herein an optical glucose biosensor for glucose monitoring, where a fluorescent enzymatic hydrogel is incorporated with fluorescein, polyethylene glycol and glucose oxidase. Glucose oxidase catalyzes oxidation of glucose to gluconic acid that protonates pH-sensitive fluorescein motif in hydrogel and thus significantly varies its fluorescence which was optically measured and correlated with glucose concentration. Therefore, fluorescence “turn-off” response is observed when glucose responsive hydrogel is exposed to glucose.

The development of fluorescent hydrogel further encouraged to develop a fluorescent hydrogel fiber as a potentially optical injectable glucose biosensor for continuous in-vivo glucose monitoring. Both glucose-responsive hydrogel sensor and glucose-responsive hydrogel fiber shows good sensitivity and reproducibility, with the fiber showing much faster response time for glucose detection. The developed glucose biosensing technique has great potential in combating diabetics.

Chapter 1

Introduction

1.1 Diabetes and Glucose detection

Diabetes

Diabetes is a metabolic disorder and a major world health problem. As stated by International Diabetes Federation, there are over 285 million people worldwide living with diabetes in 2010. This number is projected to double in 2030. Currently, the top five nations in terms of diabetic patients are China (92.4 million), India (50.8 million), US (26.8 million), Russia (9.6 million), and Brazil (7.6 million), spanning both developing and developed countries and covering diverse geographic areas. Due to the extremely large financial burden caused by diabetes and its serious complications, glucose detection is becoming important in battling diabetes and reducing financial loss [1]. Additionally, the US market for diabetic monitoring reached $9.1 billion in 2010. Therefore, glucose detection has profound social and economic implications.

One of the important functions of pancreas in human body is to produce insulin which regulates blood glucose levels in the body. Diabetes is caused due to lack of insulin secretion by pancreas (Type I) or when the cells in the body become resistant to insulin (Type II). Both low glucose levels and high glucose levels in the body are dangerous, thus it is very important to maintain body blood glucose levels. Though the administration of insulin and control of diet have allowed

patients to lead nearly normal lives, rigorous maintenance of glucose levels is necessary to slow the progression of long-term complications associated with diabetes [2, 3].

Two major situations that deal with diabetes are hyperglycemia and hypoglycemia. These two are further known for different complications. Hyperglycemia is a situation where the body glucose level falls below the normal required level. It brings down patient's blood pressure level and leads to dizziness, shaking, sweating, and sometimes patient fall unconscious, which has to be taken care immediately. On other hand hyperglycemia is a situation where the body glucose level rises way more than the normal glucose level (i.e., more than 180 mg/dl). Hyperglycemia is also called Diabetes, which when not taken care in time leads to death.

Although there is no cure for the disease, the only key for the treatment is tight glucose control substantially reduces morbidity and mortality among diabetes patients. Therefore, there is urgency for glucose detection because of serious health complications of diabetes.

Glucose Detection

Currently strip-based electrochemical glucose self-testing systems rely on glucose oxidase (GOx)-based or glucose dehydrogenase (GDH)-based glucose detection methods. Although they dominate the market and are widely used by diabetes

patients at home, the pain associated with the fingerstick can deter patients from frequent monitoring [4], resulting in a poor approximation of blood glucose variations [1(a)]. As a result, there is an urgent need to develop non-invasive or minimally invasive methods for frequent or continuous monitoring of blood glucose. Therefore, enzyme-based amperometric continuous glucose monitoring systems were first developed. Continuous *ex vivo* and *in vivo* monitoring of blood glucose was first proposed in the 1970's and 1980's, respectively [5]. *In vivo* continuous glucose monitoring would generate real-time data about the change of blood glucose levels. However, the development of reliable implantable glucose sensors is still very challenging. This is due to undesirable interactions between the implanted device and the biological medium cause rapid deterioration of amperometric sensor performance upon implantation [1(a)]. In addition, due to biofouling of the electrode surface by proteins and coagulation factors and the risk of thromboembolism, most continuous detectors do not measure blood glucose directly, and their stability and calibration to blood glucose levels have proven difficult [1(b)]. Recently, a tissue glucose amperometric sensor implanted for more than one year in an animal was reported, with promising results [6]. However, the need for surgical implantation of the device and potential adverse medical effects may limit its use by diabetic patients at home. Despite extensive research efforts in these areas over past years, no FDA approved reliable method is presently available for continuous long-term implantable tissue glucose monitoring [1(a)]. Alternatively, extensive efforts have been devoted over the past decade toward the design of subcutaneously implantable needle-type electrodes for short-term

measurement of glucose concentrations in interstitial fluid, which reflect the blood glucose level [1(a), 7]. To date, several needle-type continuous glucose sensors have been approved by U.S. FDA, including the Guardian REAL-time continuous glucose monitoring system, Dexcom SEVEN Plus, Medtronic's MiniMed Paradigm® REAL-Time System and iPro2. Although these 'under-the-skin' devices can display updated real-time glucose concentrations every one to five minutes, their lifetime is very limited (3-7 days) due to similar biofouling problems, as well as poor stability associated with the needle-type sensor [8]. Subcutaneous continuous glucose monitoring can also be achieved without direct contact between the interstitial fluid and transducer by using the microdialysis technique [9]. For example, glucoDay (Menarini, Florence, Italy) and SCGM (Roche, Mannheim, Germany) are based on a microdialysis technique. However, major challenges to long-term subcutaneous continuous glucose monitoring, including biocompatibility, linearity, calibration, specificity, drift and long-term stability, and miniaturization, still hamper their routine clinical usefulness. Other methods such as transdermal glucose sensor were also proposed as an alternative. Approved by the U.S. FDA, the GlucoWatch Biographer (Cygnus, Inc., Redwood City, CA, USA), is a watch-like electrochemical device based on transdermal extraction of interstitial fluid by reverse iontophoresis. However, it has not gained wide acceptance due to long warm-up time, false alarms, inaccuracy, skin irritation, and sweating. As a result, the GlucoWatch was withdrawn from the market in 2008. Thus, new glucose-sensing strategies need to be developed. Non-invasive and minimally invasive optical approaches thus come out as an alternative [10]. To

date, different optical techniques such as polarimetry [11], Raman spectroscopy [12], infrared absorption spectroscopy [13], photoacoustics [14], and optical coherence tomography [15] have been studied for non-invasive glucose detection. However, due to the complexity of tissues, reliable non-invasive optical glucose measuring methods are still unavailable. Another widely used optical glucose sensing method is fluorescence-based glucose sensor, which holds great promise for continuous glucose monitoring in vivo.

1.2 Fluorescence-based glucose monitoring

Fluorescence-based glucose monitoring method depends on principle of fluorescence, where fluorescence is the emission of light by a substance that absorbs light or other electromagnetic radiation. A fluorophore is a molecule that absorbs light at a particular wavelength and re-emits energy at a different wavelength. On addition of glucose to the fluorescence based glucose biosensor, the fluorescence either increases or decreases from the baseline with respect to the glucose concentration.

In the past decades, various fluorescent glucose sensors have been developed based on the use of concanavalin A (Con A), GOx, GDH, hexokinase, apo-glucose oxidase, glucose binding protein, and boronic acid derivatives as the sensing elements in conjunction with various fluorophore reporters such as green fluorescence protein (GFP) through different sensing mechanism such as the measurement of the photo-induced electron transfer (PET) induced by glucose,

the measurement of fluorescence resonance energy transfer (FRET) induced by glucose-induced conformation change of proteins/enzymes, or glucose–induced changes in intrinsic fluorescence of enzymes [16, 17].

1.2.1 Concavalin A

Concanavalin A (Con A) is one of the best characterized lectins, and it is mannose and glucose specific [18]. Con A is known for its affinity for polysaccharides. The lectin Con A is a glucose-mannose binding plant metalloprotein isolated from the jack bean [19] which requires both calcium and manganese cations to complete the formation of a saccharide binding site per protomer [20]. It is used in different types of glucose monitoring because of its four binding sites and reversible binding of sugars.

The fluorescence based approaches for glucose detection using Concavalin A (Con A) is mainly due to the competitive bind between the four glucose binding sites of Con A and fluorescein-labeled analogue [16, 21]. For example, a turn-on non-invasive fluorescence based glucose biosensor, potentially suitable for subcutaneous implantation – like a tattoo using concanavalin A (Con A) incorporated with photopolymeriszed poly(ethylene glycol) (PEG) hydrogel has been reported. Here α- acryloyl, ω-N- hydroxysuc-cinimidyl ester of PEG-propionic acid are used to chemically conjugate physically immobilized fluorescein isothiocyanate dextran (FITC-dextran) and chemically immobilized pendant

tetramethylrhodamine isothiocyanate concanavalin A (TRITC-Con A) into a PEG hydrogel [3]. Fluorescence increasing as a result of glucose fluorescein-labelled dextran and concavalin A binding is shown in Figure 1. Upon glucose addition, the glucose preferentially binds with Con A and releases fluorescein-labelled dextran, thus increasing its fluorescence intensity when compared to fluorescein-labelled dextran complexed with ConA. Higher intensity of fluorescence emission will be observed with increase in glucose concentration [22].

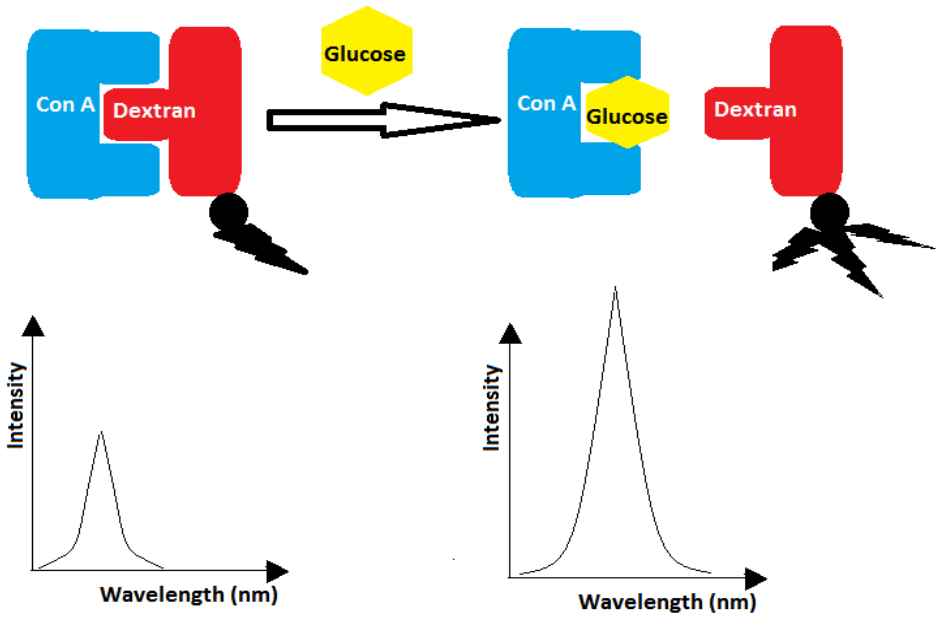


Figure 1: *Fluorescence optical detection based on binding affinity.*

On other hand, ConA fluorescence-based glucose sensors based on fluorescence emission between ConA as donor and fluorescein labeled dextran as acceptor

occurs as a result of FRET (foster resonance energy transfer), when they are within foster radius of each other [16, 19].

By evaluating FRET-induced fluorescence emission intensity of fluorescein-labelled dextran, glucose concentrations can be monitored. This is due to the increase in distance between glucose and ConA more than forster distance. Thereby this results in decrease in FRET-induced fluorescence emission [23, 24].

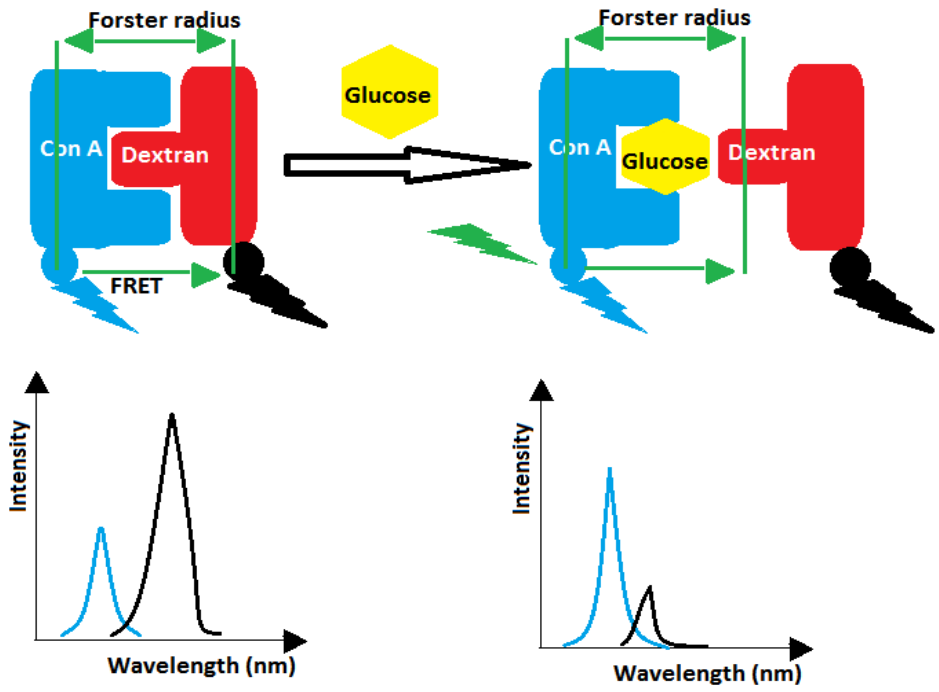


Figure 2: *Fluorescence optical detection based on decrease in FRET-induced fluorescence intensity.*

Glucose concentrations can also be evaluated by monitoring changes in lifetime of the donor molecule as the occurrence of FRET between the donor and the acceptor molecules can be influenced by the lifetime of donor molecule. Increase of glucose concentration can enhance the lifetime of donor molecule to prevent decrease in FRET [16]. However, there is a need to improve the stability of ConA and also look for alternatives to Con A mainly because of its short lifetime.

1.2.2 Boronic Acid

In 1880, before boronic acid's binding ability with diols was discovered, Michaelis and Becker synthesized phenylboronic acid (PBA) [25]. The ability of borates to interact with hydroxyl compounds was resolved when Kuivila and his group discovered that polyols and saccharides dissolve in boronic acid solutions [26].

There have been several boronic acid based glucose sensing membranes reported in the literature [27], and also it has been well-understood that boronic acid derivatives can form stable complexes with carbohydrates using either 1,2- or 1,3-diol groups [21]. In this regard, boronic acid based glucose sensors are designed mainly based on their ability of strongly and reversibly binding with diol moieties. In addition, boronic acids are synthetic receptors which could be incorporated into polymers [28, 29].

A glucose sensor based on photoindused electron transfer (PET) is observed in anthracene phenylboronate compounds. This dye is designed for fluorescent

saccharide measurement, and has weak or no fluorescence in the absence of saccharides [30, 31] due to the unshared electron pair of nitrogen atom which occupies lowest excited energy to suppress the fluorescence. In most of the solution based techniques when glucose reacts with boric acid, it increases the acidity of the solution [32] and changes boronic acid-appended fluorophore upon glucose binding by “switching on” fluorescence. The increase of fluorescence is due to the fact that the electron deficient boron atom had bound to electron rich nitrogen [33, 34] which could be studied using fluorescence spectroscopy [35, 36]. Figure 3 explains the basic mechanism of glucose sensing using fluorescent boronic acid [30, 31].

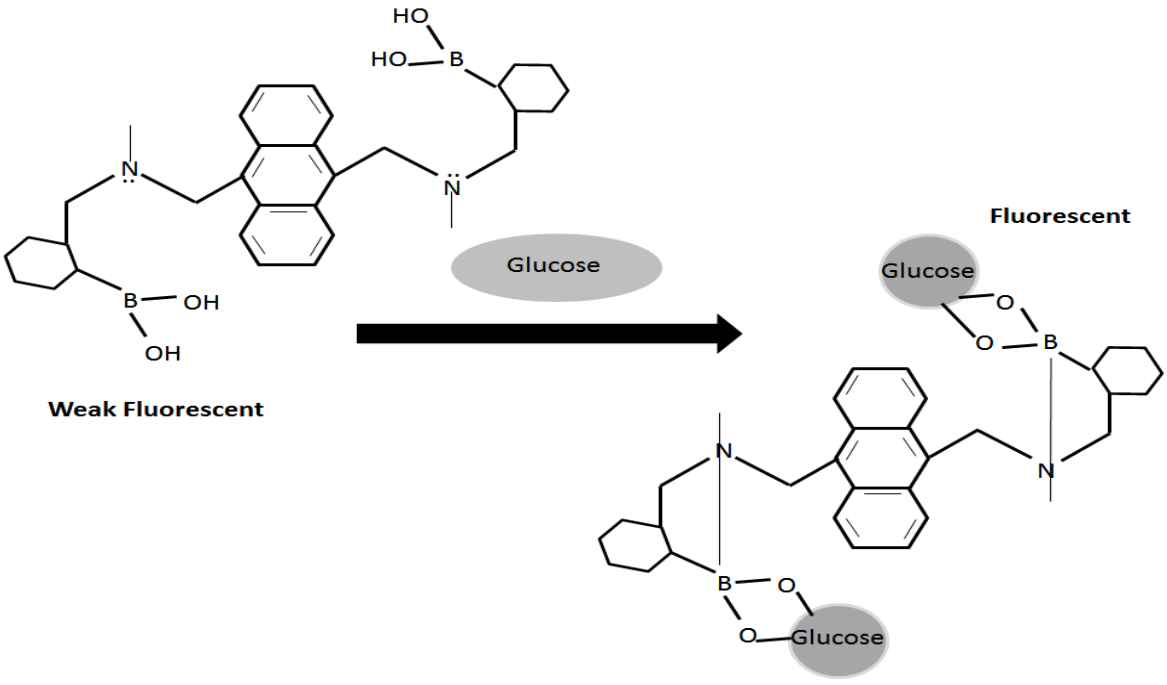


Figure 3: *Boronic acid derivatives based fluorescence sensing mechanism for glucose.*

Potentially, there are several ways to approach continuous, noninvasive glucose monitoring using boronic acid derivatives, including near-infrared (NIR) spectroscopy [37, 38], contact lens type of sensors [39] and implanted sensor devices [19]. Except for the direct spectroscopic method where a fluorescence spectroscopy is used to monitor fluorescence changes in boronic acids derivative upon glucose binding, the others require the use of a sensor as the recognition and transducer unit.

One such example is from injectable hydrogel microbeads for fluorescence based in vivo continuous glucose monitoring [40], where fluorescent hydrogel fiber was prepared for glucose sensing. The fluorescence intensity of glucose-responsive fluorescent monomer increased depending on glucose molecules existence, followed by coupling it with diboronic acid and hydrophilic spacer by polymerization using polyethylene glycol (PEG) and acrylamide (AAm). Finally it was immobilized on polyacrylamide hydrogels possessing sparse and flexible matrix [40]. A reversible reaction between glucose responsive fluorescent monomer and glucose was observed where with increase in glucose concentration the fluorescent intensity increased and vice versa. Though it has credible results on one hand, it does not have substrate specificity on the other hand.

Today, boronic acid – based glucose sensors can be used for both non-invasive and continuous glucose monitoring which indeed is an immense progress, specially the development of water soluble and photochemically stable fluorescent boronic acid derivatives. However, the short excitation and emission wavelengths

for most of these fluorescent boronic acid derivatives leave much to be desired. More work is needed to address these practical problems in order to develop a better glucose sensing system.

1.2.3 Glucose binding protein and Hexokinase

Binding proteins recognize only their respective substrates where the *E. coli* glucose binding protein (GBP) manly recognizes glucose for which it is employed as one of the glucose receptive molecule. These are soluble proteins found in periplasmic space of gram negative bacteria (e.g., *E. coli*), containing similar structure of sugar and other ligand binding proteins. Transporting molecules to the membrane receptors is the key role played by the binding proteins [41, 42]. These binding proteins are not enzymes and there is no requirement of additional ingredient for proceeding sensing mechanism. Thus they are considered as reagentless biosensors.

Glucose binding proteins is used as a glucose sensor because it has a hinge like structure where a single polypeptide chain folds into two domains. The structure is explained in Figure 4 [43]. Because of their large intrinsic flexibility, on glucose binding the protein changes from an “open” ligand-free structure to a “closed” ligand-bound structure resulting in fluorescence change of a polarity sensitive fluorescent probe [44, 45].

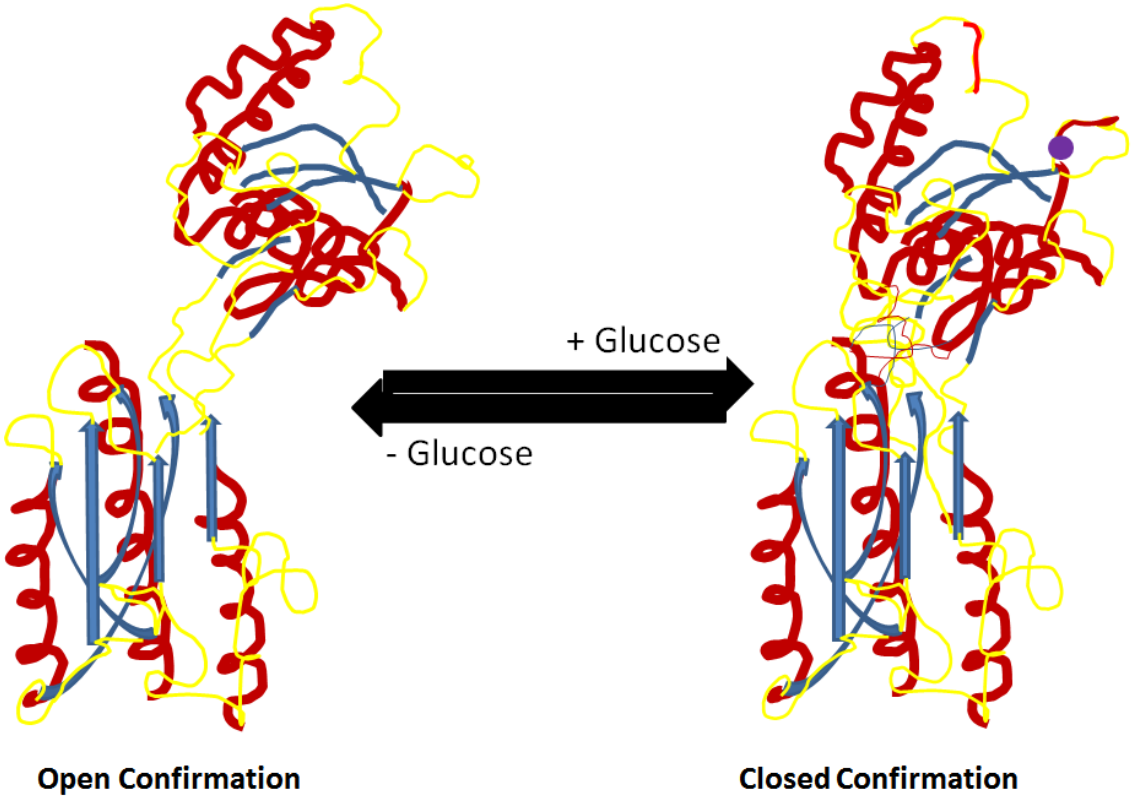


Figure 4: *Open and closed ribbon structures of glucose binding protein (GBP) depicting conformational change.*

Equivalent change in response to ligand binding can be indirectly detected. Corresponding optical results are micromolar sensitive to glucose allow us to monitor glucose levels, either through changes in FRET between donor-acceptor pairs on the protein or by change in fluorescence due to environmentally sensitive fluorophore [46, 47].

A hexokinase is an enzyme that catalyzes the transfer of the γ phosphoryl group of ATP to the hydroxyl group at position C6 of glucose. For such activation a divalent metal ion such as Mg^{2+} or Mn^{2+} is required.

$$\text{ATP} + \text{D-glucose} \rightarrow \text{ADP} + \text{D-glucose-6-phophate}$$

Several groups reported that the hexokinase enzyme has intrinsic fluorescence with excitation and emission wavelengths to be λ_{ex}=295 nm and λ_{ex}=330 nm respectively, attributed to tryptophan residues in the protein-which exits in a dimeric form. Each monomer subunit of the enzyme bifurcates into two lobes, as shown in Figure 5.

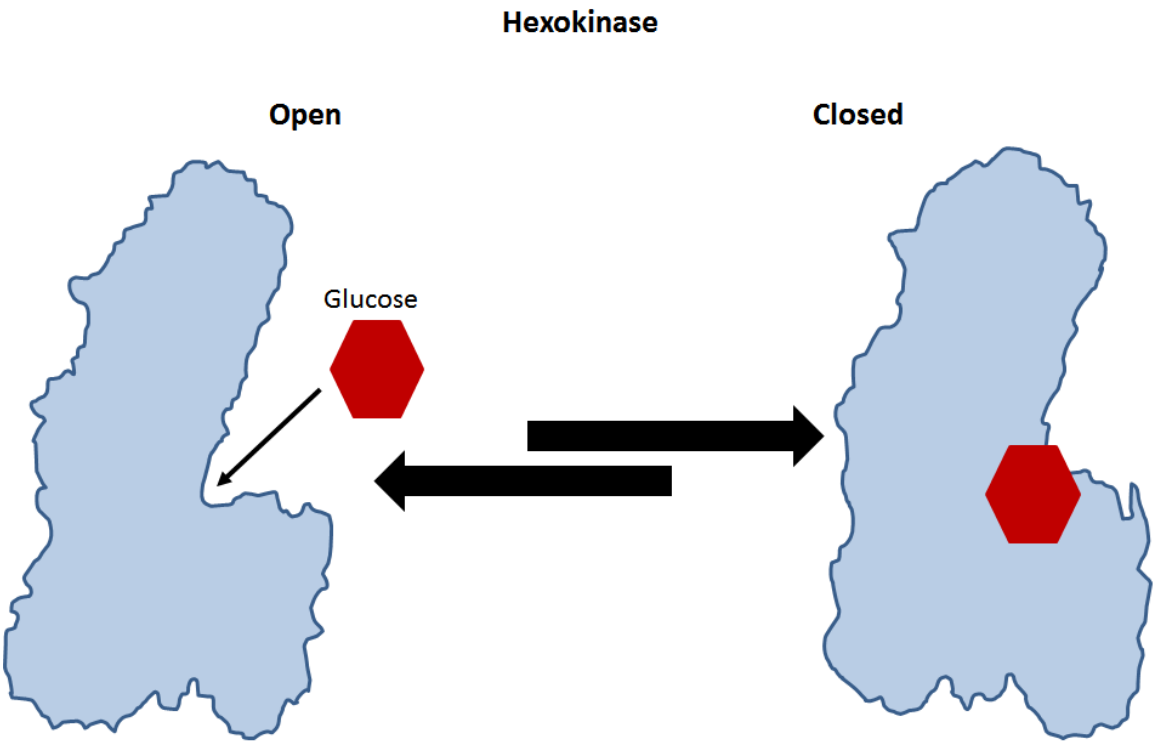


Figure 5: *Closed conformation of Hexokinase induced by glucose binding.*

Binding of glucose at the active site in the cleft leads to a conformational change in the enzyme, such that the two lobes move closer together and the glucose is almost surrounded by protein [48]. Each subunit of yeast hexokinase has four tryptophan residues as shown in Figure 6, domain 1 & 2 are two surface residues, domain 3 is a glucose quenchable residue at the cleft and domain 4 buried. Monitoring the fluorescence change on addition of glucose will thus provide information about glucose concentrations [48].

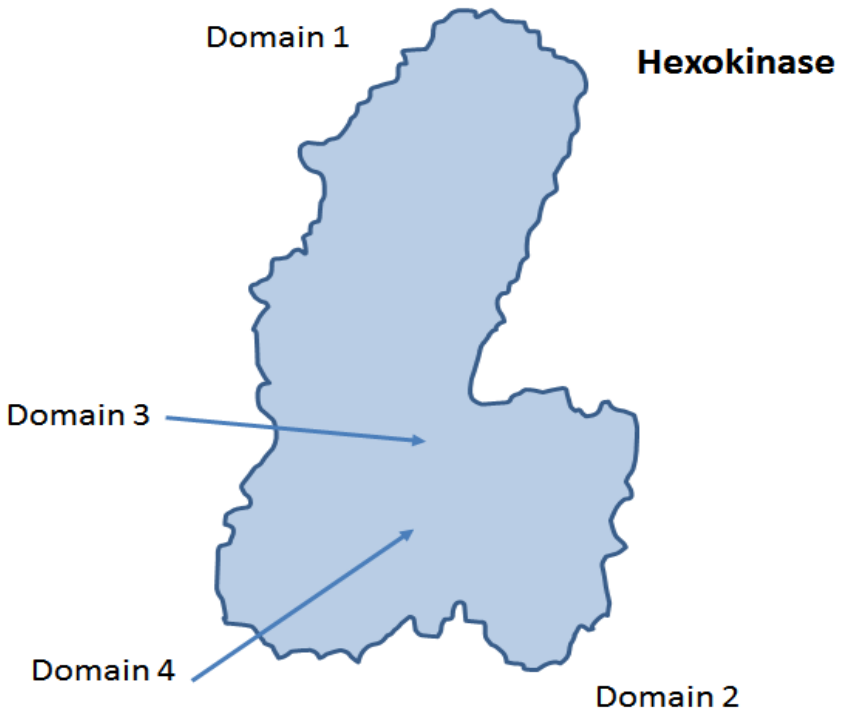


Figure 6: *Four tryptophan residues of yeast hexokinase.*

1.2.4 Glucose oxidase

Glucose oxidase is a small stable enzyme found in honey and is a natural preservative. It is a dimeric protein with 3D structure. Due to its high selectivity to glucose, significant pH range, ionic strength, and temperature, glucose oxidase detection yields maximum specificity for glucose estimation and has been widely applied in glucose detection [49-56].

Glucose oxidase is a glucose specific enzyme that plays the role of glucose receptor molecule and binds with fluorophore allowing energy transfer between flavin group of enzyme and the fluorescein [57]. Thus glucose oxidase is utilized as a glucose receptive molecule where the enzyme is immobilized in a polymer [58-68], which catalyzes oxidation of glucose to gluconic acid and hydrogen peroxide. In this process, flavin adenine mononucleotide (the enzyme's redox cofactor) is converted to flavin adenine dinucleotide [1].

$$\text{Glucose} + \text{Oxygen} \xrightarrow{\text{Glucose Oxidase}} \text{Gluconolactone} + H_2O_2$$

$$\text{Gluconolactone} + H_2O \longrightarrow \text{Gluconic acid}$$

In recent years many researchers started working on immobilizing glucose oxidase on various surfaces [69-72] for fabrication of glucose biosensors based on the concept of enzyme electrodes [73].

Looking at the research in the past 5 years there could be seen different techniques adopted by researches to come up with fluorescence based glucose biosensor with an enzyme electrode. They commonly use the enzyme-glucose oxidase as their glucose receptive molecule. On addition of glucose the enzyme catalyzes by protonating the fluorophore, resulting in the fluorescence change of the biosensor with respect to glucose concentration. It could be either a “turn-on” response of fluorescence or a “turn-off” response depending on the technique adapted.

For example, an optical glucose biosensor was recently fabricated by encapsulating glucose oxidase in silica gel via sol-gel method with $Ru(bpy)_3Cl_2$ as a luminescent oxygen transducer, for monitoring glucose concentrations in body blood and urine samples [74]. On addition of glucose ranging from 2 mM to 18 mM, oxidation occurs due to the enzyme catalysis. An increase in fluorescence of $Ru(bpy)_3Cl_2$ was observed due to oxygen consumption [74].

As mentioned above the enzyme catalyzes oxidation of glucose to gluconic acid and hydrogen peroxide. A “turn-on” response is observed in this case where the fluorescence intensity of the biosensor increases from its normal line, due to oxygen consumption. The fluorescent glucose biosensors can also be developed using the enzymatic product of hydrogen peroxide. Recently a glucose oxidase biosensor was developed based on Fenton reaction triggered molecular beacon cleavage. Initially a low fluorescence was observed due to high quenching efficiency in molecular beacons, but on addition of glucose, hydrogen peroxide is generated with the oxidation of glucose by the enzyme. *OH was produced through

Fe^{2+} catalyzed Fenton reaction and then triggered the cleavage of molecular beacon, resulting in increase of its fluorescence due to complete separation of the fluorophore from the quencher [75]. Thus a “turn-on” response is observed due to hydrogen peroxide generation.

Similarly many techniques, including fluorophore and GO_x functionalized gold nanoclusters as probe, GO_x immobilized on layer-by-layer fluorophore films, etc., are also developed for glucose detection based fluorescence change [76].

Objective

Even though a variety of fluorescence biosensors based on different materials have been reported for the detection of glucose, a biocompatible fluorescence-based glucose sensors with good sensitivity, excellent selectivity, reproducibility and stability, as well as low cost, are always highly demanded for quantitative determination of glucose. The major effort of this research focuses on the design of novel biocompatible glucose-responsive fluorescent hydrogel towards glucose detection. We are aiming at developing glucose sensors which have potential application for in vivo continuous glucose detection in a simple, sensitive, selective and cost-effective way.

Chapter 2

Experimental Section

2.1 Reagents and Equipment

Glucose oxidase (GO_x), fluorescein-o-acrylate (FOA), poly ethylene glycol diacrylate (PEGDA, M.W.=250) and glucose were obtained from Sigma-Aldrich. Tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from Bio-Rad. Solidum chloride, monosodium phosphate, disodium phosphate are acquired from Fisher. All solutions were prepared with deionized (DI) water (18.2 MΩ-cm) from a Barnstead DI water system. As human body fluid (e.g., blood) is a buffered solution consisting of NaCl ranging from 100 mM to 200 mM, 20 mM pH 7.4 phosphate buffer solution with or without 150 mM NaCl were used in the study.

Absorption spectra were performed on a Varian Cary 50 UV-Vis NIR spectrophotometer, and fluorescence emission spectra were recorded on a Varian Carry Elipse Fluorescence Spectrophotometer. Microplate reader (Biotek, synergy HT) was employed to measure the fluorescence of hydrogels in multi-well plate.

2.2 Measurement of pH-dependent fluorescence of fluorescein-o-acrylate in phosphate buffer solution

To study the pH-dependent fluorescence of fluorescein-o-acrylate in phosphate buffer solution, 500 μg/mL of fluorescein-o-acrylate was dissolved in 20 mM phosphate buffer solution with different pH values (pH 5.8, pH 6.6, pH 7.4, and pH 8.2). The corresponding UV-vis absorption and fluorescent emission spectra (λ_{ex}=489 nm) were collected.

2.3 Preparation of glucose-responsive fluorescent hydrogel

To form a hydrogel, fluorescein-o-acrylate (FOA) and polyethylene glycol diacrylate (PEGDA) mixture containing GO_x was polymerized with TEMED and APS as accelerator and initiator, respectively. The reaction is a vinyl addition polymerization initiated by a free radical-generating system. Chemical polymerization is initiated by APS (initiator) and TEMED (accelerator) which accelerates the formation of free radicals from persulfate thus catalyzing the polymerization. The persulfate free radicals convert FOA and PEGDA (both serving as monomers) to free radicals which react with inactivated monomers to start the polymerization. The elongating polymer chains are randomly crosslinked by PEGDA (also serving as crosslinker), resulting in a hydrogel with a characteristic porosity which depends on the polymerization conditions and monomer concentrations. Meanwhile, GO_x was entrapped in the hydrogel simultaneously. FOA endows the hydrogel fluorescence and PEG introduces good biocompatibility to the hydrogel, while GO_x endows the hydrogel glucose

responsiveness. Therefore, the as-prepared glucose-responsive fluorescent hydrogel provides an excellent biosensing platform for glucose monitoring.

To prepare a typical 100 µL hydrogel, 49.33 µL 20 mM pH 7.4 sodium phosphate buffer solution, 20 µL of FOA solution (500 µg/mL in the same phosphate buffer solution), 10 µL of liquid PEGDA, 10 µL of GO_x solution (100 mg/mL in 20 mM pH 7.4 buffer), 0.66 µL of TEMED were mixed first. Then 10 µL of 1 wt% APS solution (in water) was added to trigger the polymerization.

Once all chemicals are added, immediate vigorous mixing is required until polymerization starts. Within half a minute hydrogel was formed and the final GO_x concentration in hydrogel is 10 mg/mL. GO_x loading in hydrogel can be changed using different stock GO_x solution. To prepare any other volume of hydrogel, the same volume ratio is applied. A schematic drawing of the crosslinked PEG-FOA hydrogel with GO_x entrapped in the hydrogel matrix was shown in Figure 7.

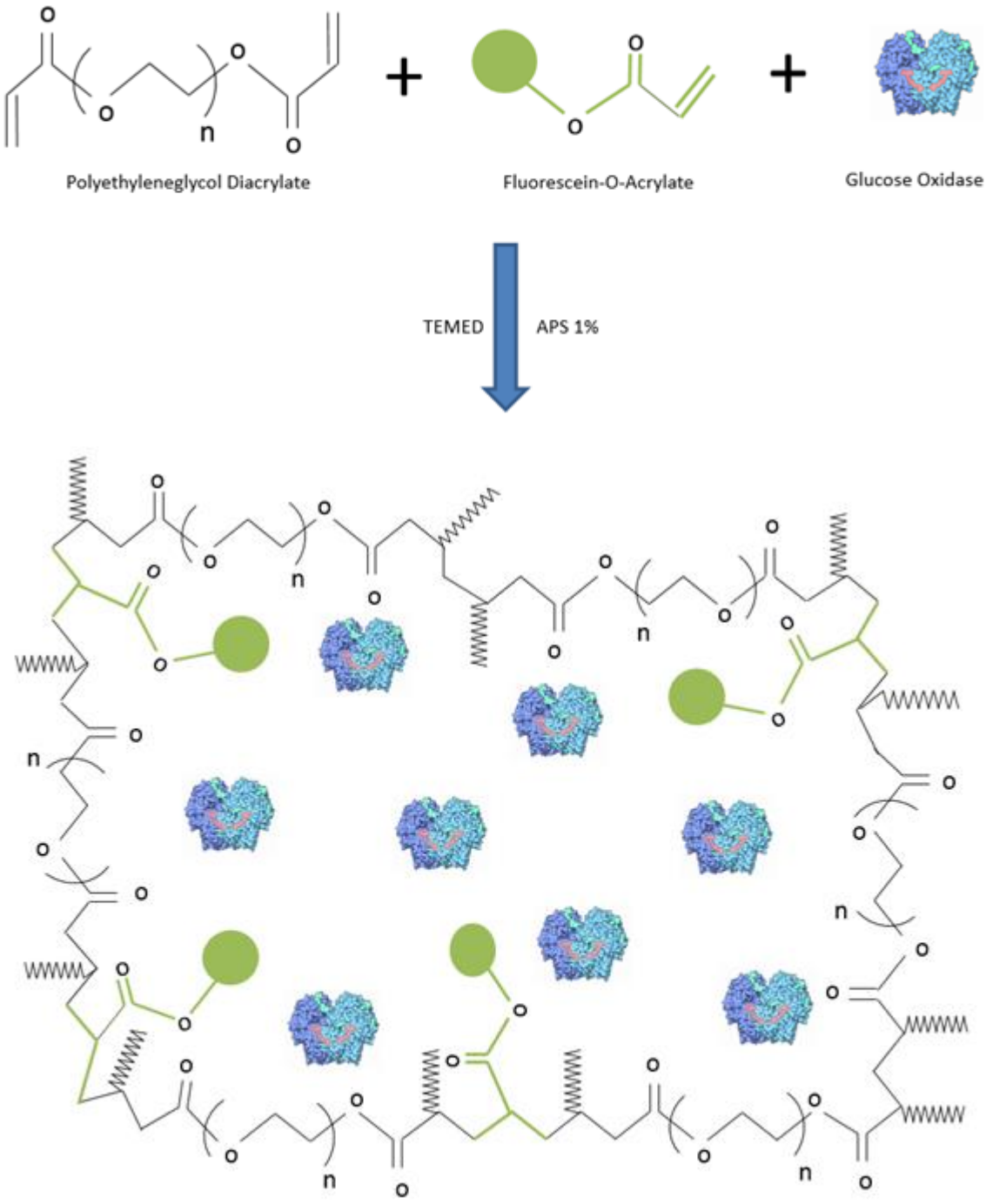


Figure 7: *Schematic representation of the cross-linked glucose-responsive fluorescent hydrogel (not in scale).*

The as-prepared hydrogel appears to be pale yellow in color to naked eye under white light (Figure 8-A), while it displays a high green fluorescence under UV-light of 365 nm wavelength (Figure 8-B).

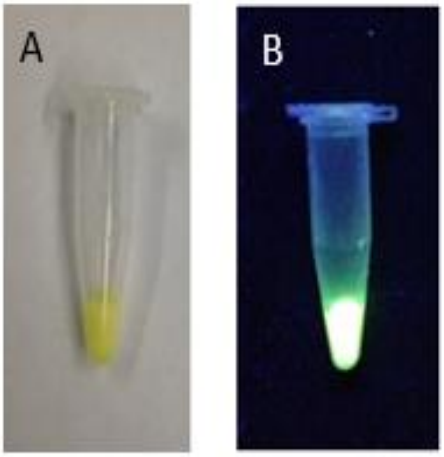


Figure 8: *(A) An optical image of the as-prepared hydrogel under white light; (B) A fluorescence image of the as-prepared hydrogel under 365 nm UV light.*

Chapter 3

Results and Discussion

3.1 pH-dependent fluorescence of fluorescein-o-acrylate

Fluorescein and many of its derivatives exhibit multiple, pH-dependent ionic equilibria. It has been proven that fluorescein is a pH-responsive fluorophore. In present work fluorescein-o-acrylate was used and then its pH-dependent fluorescence was investigated first. Solution based emission and absorption spectra of fluorescein-o-acrylate were collected and presented in Figure 9. One can see that the absorption and emission peaks of FOA were observed at 489 nm and 512 nm, respectively. With the increase of pH from pH 5.8 to pH 8.2, both the absorption peak intensity and emission peak intensity increase, showing a pH-dependent behavior. This study indicates that FOA can also be employed as a reporter to local pH change.

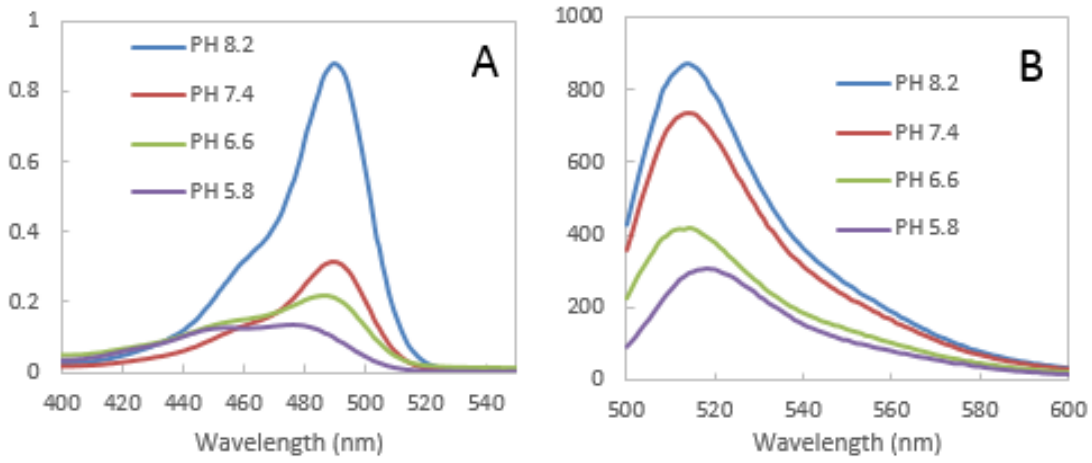


Figure 9: *UV-vis absorption (A) and emission (B) spectra of FOA in different pH buffer solution (λ_{ex}=489 nm).*

In a basic medium an open structure of FOA is observed as shown in Figure 10. With the increase of acid concentration, a spiro structure or lactone structure of FOA is observed, resulting in the reduced fluorescence. Therefore, to lower the pH results in the “turn-off” of its fluorescence.

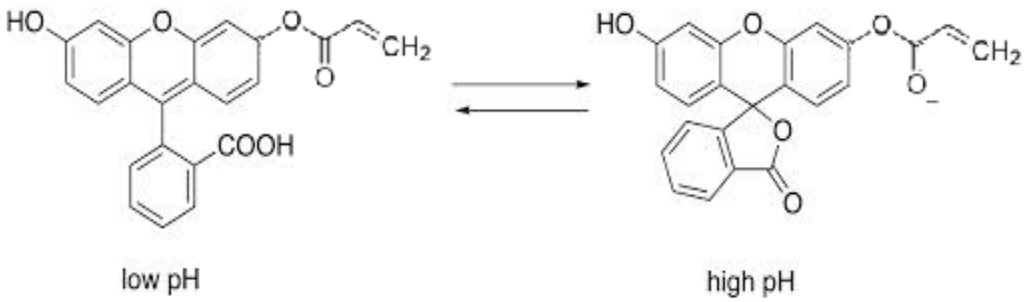


Figure 10: *The mechanism for pH-dependent fluorescence of FOA.*

3.2 pH-dependent fluorescence of the as-prepared hydrogel

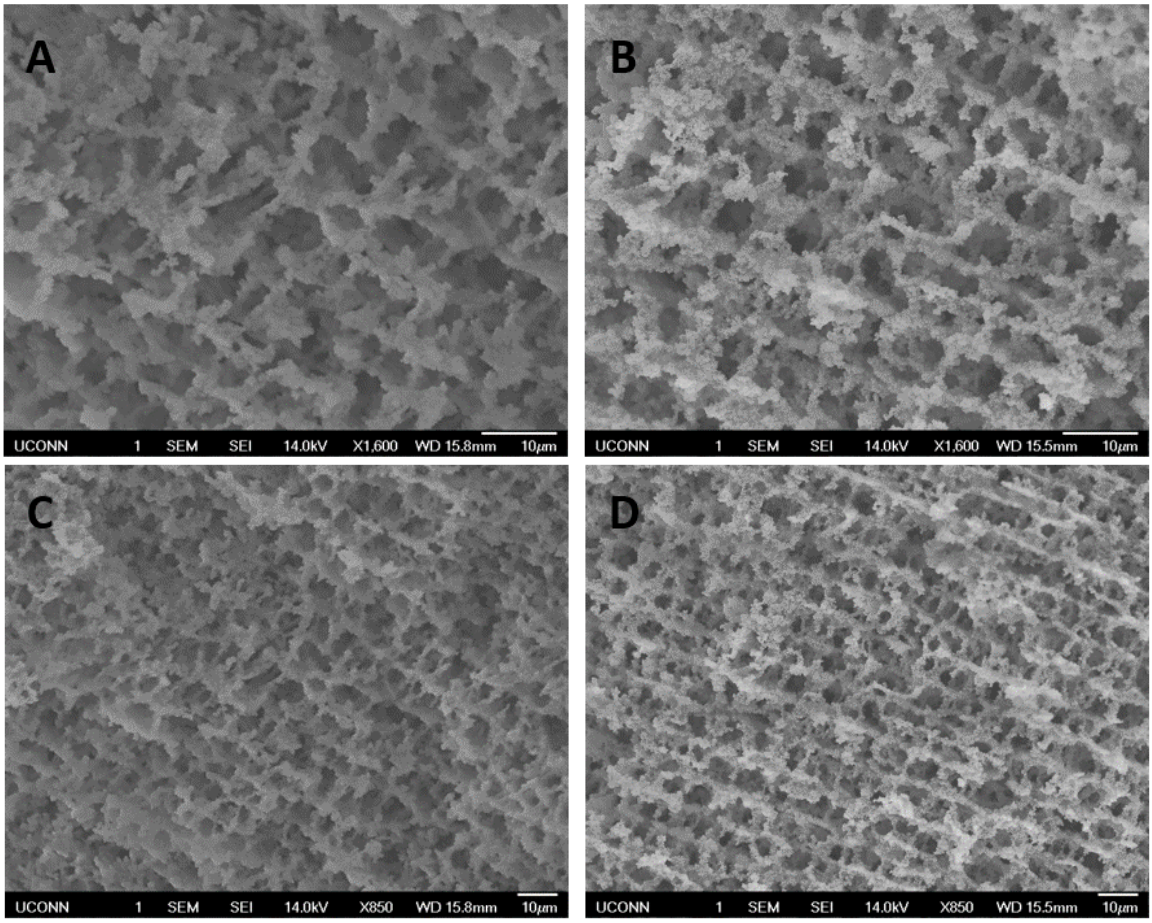


Figure 11: *SEM images of lyophilized PEG-FOA hydrogel (A,C) and PEG-FOA-GO_x (B,D) in high (A,B) and low (C,D) magnification.*

The morphology of hydrogel was first evaluated using Jeol Field emission scanning electron microscope (JSM-6335F Thermo Electron Corporation). PEG-FOA hydrogel and PEG-FOA-GO_x hydrogel were firstly prepared at room temperature and then subject to lyophilization. Figure 11 shows the corresponding SEM images of lyophilized hydrogels. As can be seen, both hydrogels show highly porous structures with pore size less than 10 µm. The incorporation of GO_x in hydrogel (Figure 11, B and D) resulted in the hydrogel scaffold consisting of smaller particles.

To investigate the pH-dependence of the fluorescence of as-prepared hydrogel, hydrogel was first prepared in multi-well plate with a volume of 150 µL/well. The same recipe described in Chapter 2 was used to prepare hydrogel in multi-well plate. Once the hydrogel was formed, fluorescence measurement was taken and the fluorescence of each hydrogel is almost same. Hydrogel in each well was then treated with 200 µL of 20 mM sodium phosphate buffer solution with pH value of pH 5.8, pH 6.6, pH 7.4 and pH 8.0, respectively. After 45 min interaction, fluorescence measurements were conducted in situ by taking the readings from a micro-plate reader. All experiments were carried out in triplicate. As shown in Figure 12, the fluorescence of the hydrogel decreases with pH decrease from 8.2 to 5.8. The observed trend is in good agreement with pH-dependent fluorescence of FOA in solution (Figure 11B).

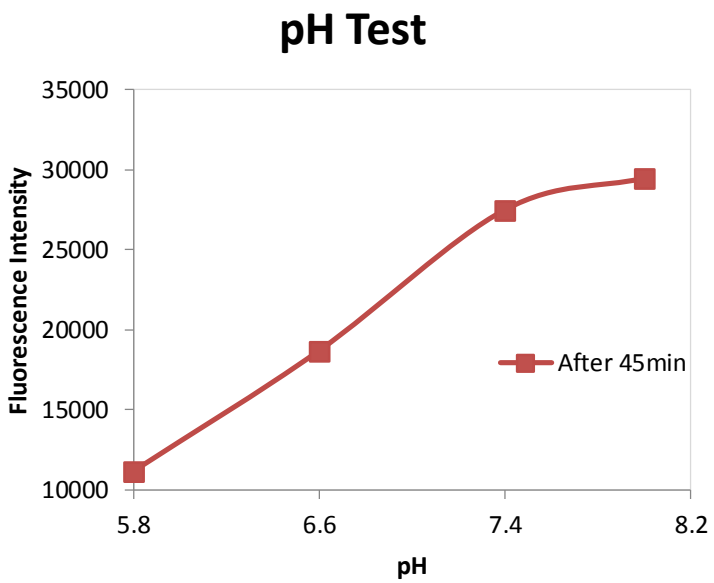


Figure 12: *Fluorescence response of the as-prepared hydrogels upon the addition of 20 mM sodium phosphate buffer solution with different pH value (pH 5.8, pH 6.6, pH 7.4 and pH 8.2). (λ_{ex}=489 nm and λ_{em}=512 nm).*

The fluorescence images of the hydrogels before and after addition of buffer solution with different pH values were also obtained under UV-light at 365 nm wavelength and presented in Figure 13. One can see that lower fluorescence intensity at acidic medium and higher fluorescence intensity in basic medium, in good agreement with the trend obtained from multiwall plate reader in Figure 13.

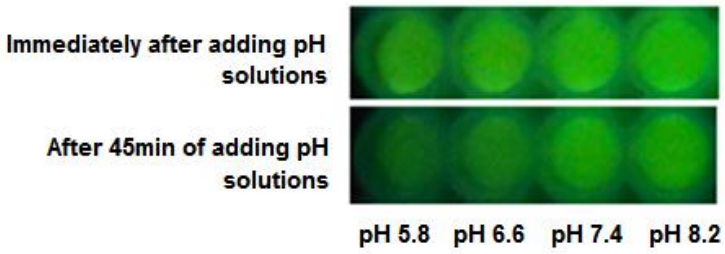


Figure 13: *The fluorescence images of the hydrogel immediately after addition of 20 mM sodium phosphate buffer solution and after 45 min of addition of 20 mM sodium phosphate buffer solution with different pH values (pH 5.8, pH 6.6, pH 7.4 and pH 8.2) under a handheld UV-lamp (365 nm).*

These studies clearly indicate that the as-prepared hydrogel possesses pH-dependent fluorescence and can respond to local pH change, which has the potential to be used as glucose biosensing material in conjunction with GO_x-based enzymatic conversion of glucose to gluconic acid.

3.3 Glucose response of the as-prepared PEG-FOA hydrogel and PEG-FOA-GO_x hydrogel

To test the ability of the hydrogel whether or not it responds to glucose, two wells of PEG-FOA-GO_x hydrogels (150 µL of hydrogel each well with GO_x final concentration of 10 mg/mL) and another two wells of PEG-FOA hydrogel (150 µL of hydrogel each well without GO_x) were prepared, and their fluorescence response to the addition of 200 µL DI water in the absence or presence of 30 mM

glucose was collected. There are four different experiments here: 1. the PEG-FOA hydrogel exposed to 200 µL DI water; 2. the PEG-FOA hydrogel exposed to 200 µL DI water containing 30 mM glucose; 3. the PEG-FOA-GO_x hydrogel exposed to 200 µL DI water; and 4. The PEG-FOA-GO_x hydrogel exposed to 200 µL DI water containing 30 mM glucose.

Upon the addition of solution to the microplate well, fluorescence was measured every 15 min using a multi-well plate reader. To have a better comparison of the fluorescence change and eliminate the very minor variation of initial fluorescence intensity of hydrogels in different wells, normalized fluorescence intensity (I/I_0) and normalized fluorescence intensity change, $(I_0-I)/I_0$, were used in Figure 14 and Figure 15, respectively. I_0 is the initial fluorescence intensity at λ_{em}=512 nm and I is the respective fluorescence reading at every 15 minutes after the addition of solution.

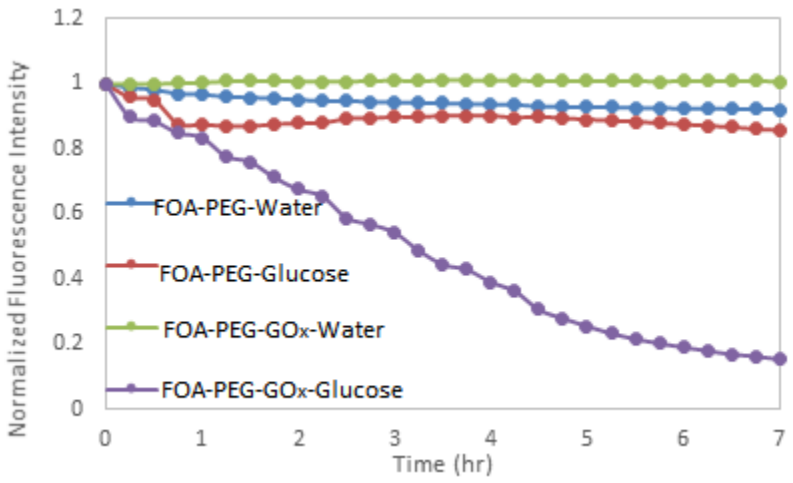


Figure 14: *Normalized fluorescence intensity vs. time for PEG-FOA hydrogel and PEG-FOA-GO$_x$ hydrogel upon the addition of DI water with or without 30 mM glucose. (λ_{ex}=489 nm and λ_{em}=512 nm).*

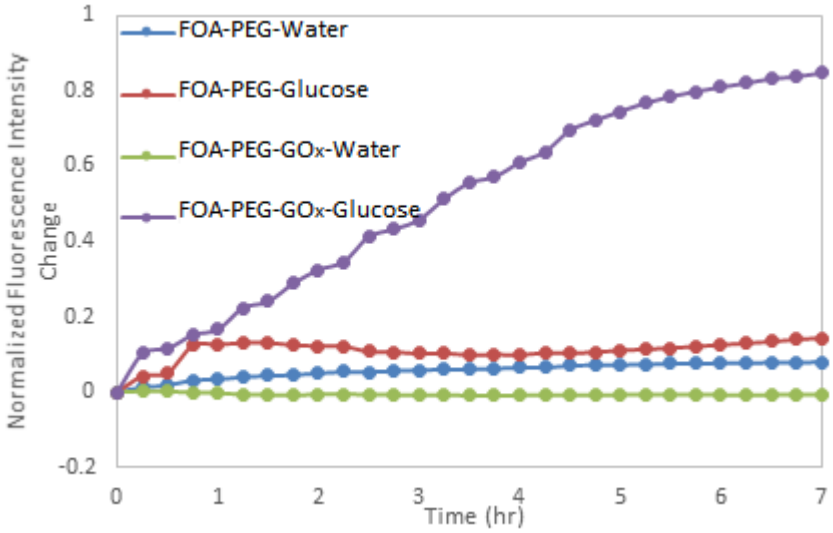
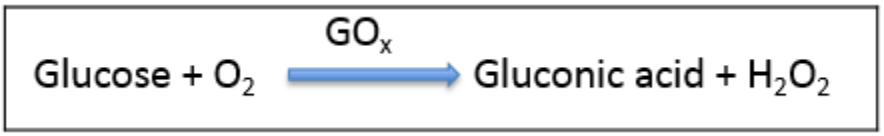


Figure 15: *Normalized fluorescence intensity change vs. time for PEG-FOA hydrogel and PEG-FOA-GO$_x$ hydrogel upon the addition of DI water with or without 30 mM glucose. (λ_{ex}=489 nm and λ_{em}=512 nm).*

The addition of DI water does not result in obvious fluorescence change for both PEG-FOA hydrogel and PEG-FOA-GO_x hydrogel. The initial drop in fluorescence intensity may be attributed to the solvent and its diffusion effect. On the contrary, the addition of 30 mM glucose solution to PEG-FOA hydrogel and PEG-FOA-GO_x hydrogel results in distinguishable response patterns. There is no obvious fluorescence intensity change observed for PEG-FOA hydrogel upon the addition of glucose due to the lack of GO_x, while the fluorescence of PEG-FOA-GO_x hydrogel gradually decreases and reaches saturation after 6 hours. The distinguished response pattern can be attributed to following mechanism. In the present of glucose, GO_x catalyzes glucose and liberates gluconic acid (glucose+O_2 $\xrightarrow{GOx}$gluconic acid+H_2O_2), resulting in the pH decrease in hydrogel. Consequently, pH-sensitive FOA responds to local pH change, resulting in the decrease of the fluorescence of hydrogel.



3.4 The effect of GO_x loading on sensing performance of PEG-FOA-GO_x hydrogel

To further investigate the effect of GO_x loading in PEG-FOA-GO_x hydrogel on the glucose sensing performance, hydrogels with different GO_x loading were first prepared with the same recipe in 20 mM pH 7.4 sodium phosphate buffer, and the final GO_x concentration is 1 mg/mL, 10 mg/mL and 20 mg/mL, respectively. Upon the addition of 200 µL of 30 mM glucose, the fluorescence of hydrogels was collected every 15 minutes over 7 hours. The corresponding normalized fluorescence intensity and the normalized fluorescence intensity change with time were presented in Figure 16 and Figure 17. As expected, the hydrogel with higher GO_x loading responded to glucose faster and then gradually reached plateau, while the hydrogel with lower GO_x loading resulted in a slower response time. In addition, the experiments were further conducted to investigate the repeatability and consistency. The low relative standard deviation of less than 5.1% for triplicate experiments in all conditions here demonstrates good reproducibility of the developed glucose-sensing hydrogel.

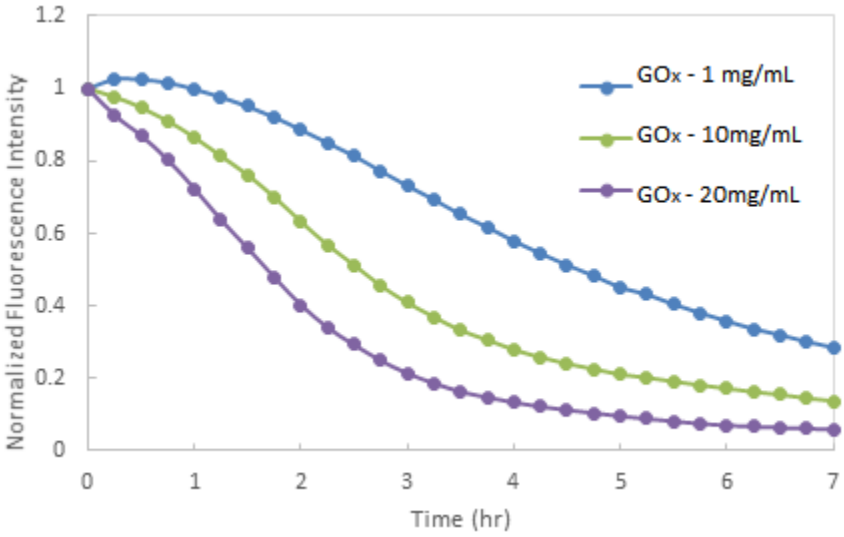


Figure 16: *The time-dependent normalized fluorescence intensity of PEG-FOA-GO_X hydrogels with different GO_X loading upon the addition of 30 mM glucose.*

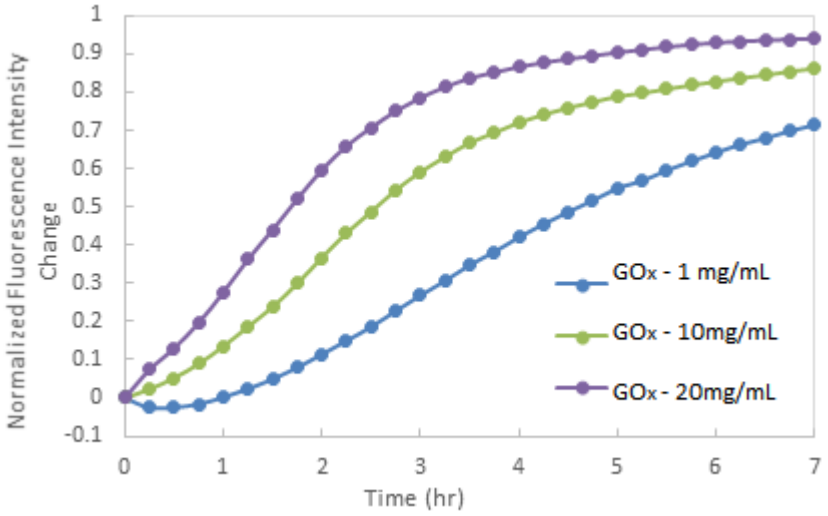


Figure 17: *The time-dependent normalized fluorescence intensity change of PEG-FOA-GO_X hydrogels with different GO_X loading upon the addition of 30 mM glucose.*

As it is observed that sodium chloride (NaCl) concentration in human body fluids varies from the range 100 mM to 200 mM, hydrogels with different GO_x loading were also prepared following in the same protocol except the use of 20 mM pH 7.4 phosphate buffer containing 150 mM NaCl. Similar experiments as previous ones were further carried out to study glucose-sensing performance of hydrogel with various GO_x loading upon the addition of 30 mM glucose. As shown in Figure 18, the normalized fluorescence intensity of hydrogel decreases with the increase of GO_X loading from 1 mg/mL to 10 mg/mL to 20 mg/mL, which may be ascribed to the faster enzymatic conversion of glucose to gluconic acid. The corresponding normalized fluorescence intensity change is also presented in Figure 21. One can see that the higher loading of GO_x in hydrogel results in a faster response and also reach saturation faster. However, given enough time (e.g., 7 hrs), all hydrogels could result in good fluorescence quenching of hydrogels. These results indicated that the presence of NaCl in hydrogel would not change the response pattern compared to the ones without NaCl. The low relative standard deviation of less than 5.1% for triplicate experiments in all conditions also demonstrates good reproducibility of the developed glucose-sensing hydrogel in the presence of NaCl.

Furthermore, we can see from the results and the normalized data above that in the presence and absence of 15 mM NaCl, hydrogels with 20 mg/mL GO_x possess a better glucose response in a faster way. However, hydrogel with 10 mg/mL GO_x also shows good response to glucose addition. To consider the effect of GO_x loading on sensing response and the cost of the use of expensive GO_x, two different hydrogels were prepared for subsequent study in glucose detection: 1.

hydrogel with 20 mg/mL GO_x prepared in buffer without 150 mM NaCl; and 2. hydrogel with 10 mg/mL prepared in buffer with 150 mM NaCl.

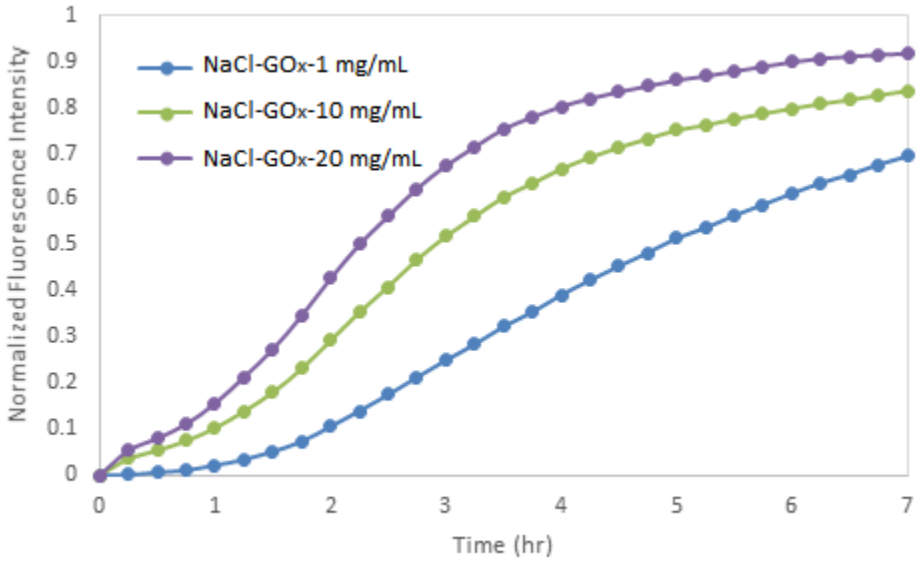


Figure 18: *The time-dependent normalized fluorescence intensity of PEG-FOA-GO_X hydrogels with different GO_X loading upon the addition of 30 mM glucose*.

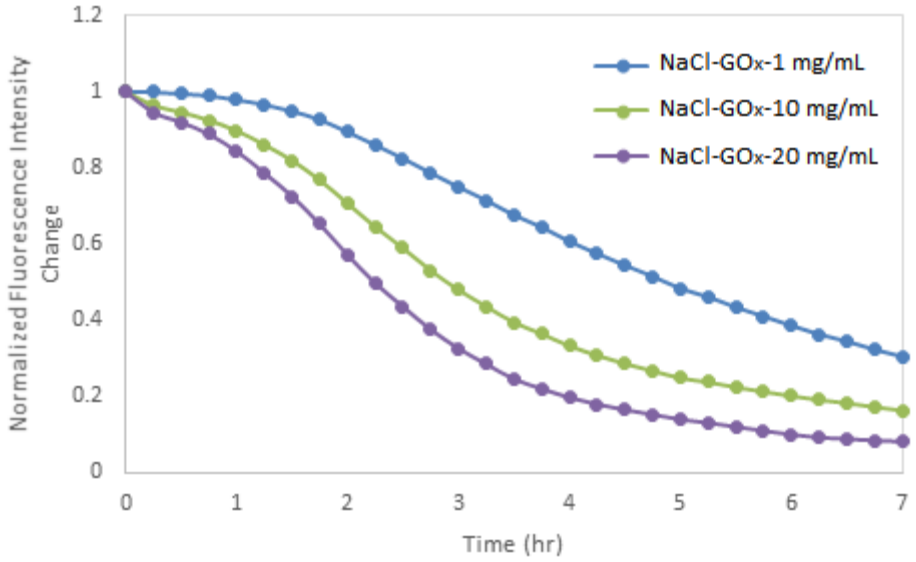


Figure 19: *The time-dependent normalized fluorescence intensity change of PEG-FOA-GO$_X$ hydrogels with different GO$_X$ loading upon the addition of 30 mM glucose.*

3.5 Glucose detection

3.5.1. Glucose sensing using PEG-FOA-GO$_x$ hydrogel prepared with 20 mM pH 7.4 sodium phosphate buffer (GO$_x$ loading of 20 mg/mL)

The real-time fluorescence detection of glucose was carried out by injection of different glucose solution (0 to 50 mM) onto the as-prepared hydrogel in microplate wells. Right after the injection of solution, the fluorescence readings were recorded as the initial fluorescence values and then the fluorescence change of each hydrogel was recorded over time. The corresponding results were shown in Figure

12 and Figure 20. The injection of water barely caused any change in the fluorescence of hydrogel. With the injection of glucose, the normalized fluorescence intensity gradually decreases over time. The injection of glucose with higher concentration results in the fluorescence quench much faster and finally caused a lower saturated fluorescence when compared to the ones injected with low glucose concentration. The normalized fluorescence intensity change (Figure 19) increases with the time when glucose concentration is fixed. In addition, at the same reaction time, higher glucose concentration results in a higher fluorescence change as expected. After 7 hours, the glucose solutions with 30 mM and 50 mM glucose can almost fully quench the fluorescence of hydrogel. This study indicates that the fluorescence of as-prepared hydrogel show concentration-dependent behavior toward glucose injection, which provides an excellent biosensing platform for glucose monitoring.

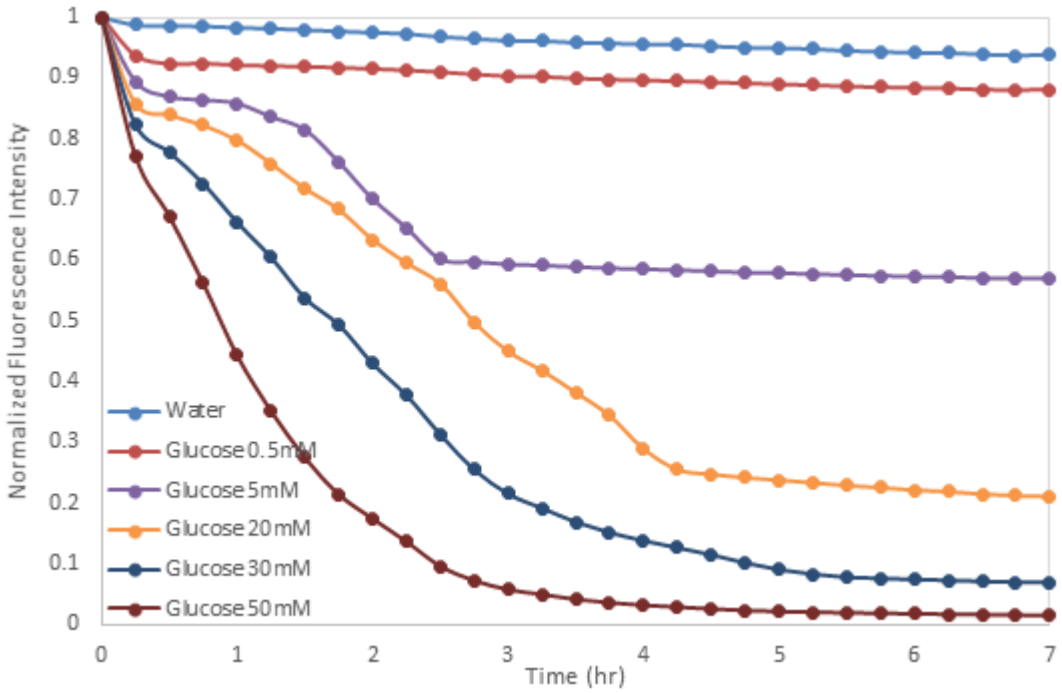


Figure 20: *The time-dependent normalized fluorescence intensity of PEG-FOA-GO_X hydrogels prepared with 20 mM pH 7.4 sodium phosphate buffer (GO_X loading=20 mg/mL) upon the addition of glucose solutions with different concentrations.*

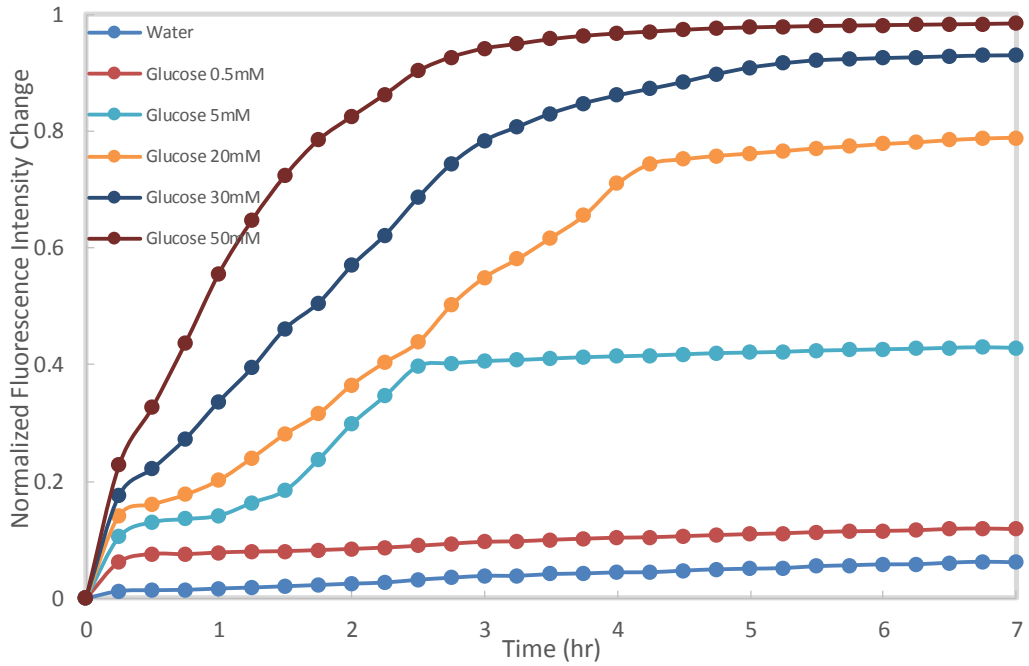


Figure 21: *The time-dependent normalized fluorescence intensity change of PEG-FOA-GO$_x$ hydrogels prepared with 20 mM pH 7.4 sodium phosphate buffer (GO$_x$ loading=20 mg/mL) upon the addition of glucose solutions with different concentrations.*

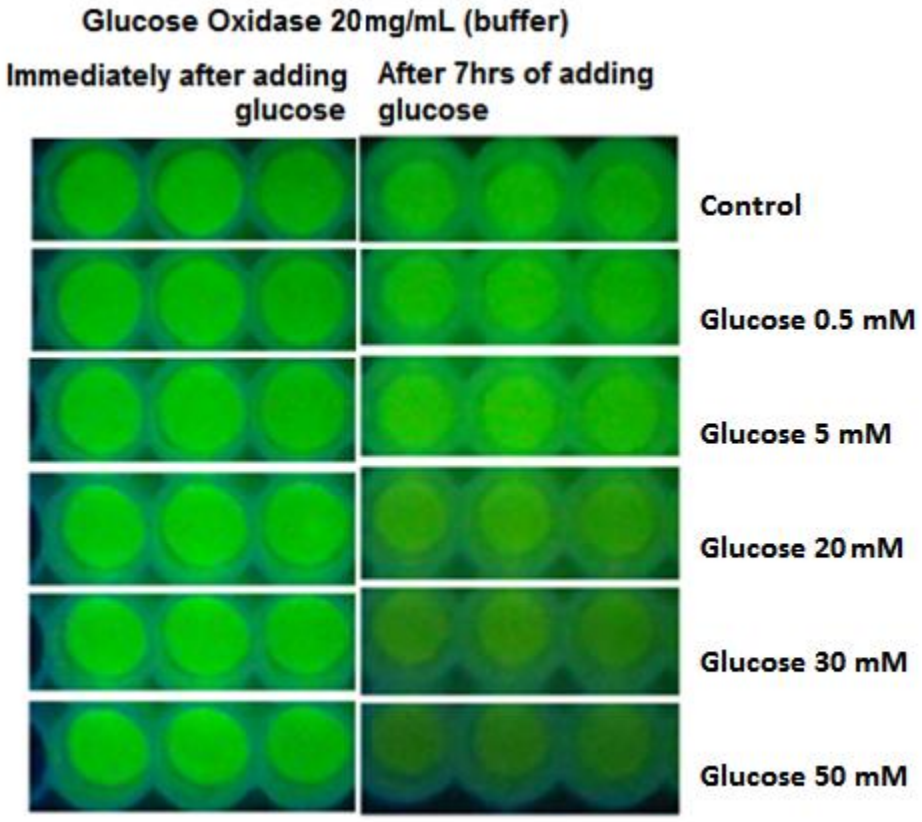


Figure 22: *The fluorescence images of PEG-FOA-GO$_x$ hydrogels prepared using 20 mM pH 7.4 sodium phosphate buffer under UV-light at 365 nm immediately and after 7hrs addition of glucose solutions with different concentrations.*

Besides the fluorescence readings using microplate reader, the fluorescence images of the hydrogel immediately and after 7 hours addition of different glucose solution (0 mM glucose to 50 mM glucose) were also obtained under UV-light at 365 nm wavelength and presented in Figure 22. One can see that the initial fluorescence of hydrogels is relatively uniform. Upon the addition of water, there is no obvious fluorescence difference in the hydrogels, but the addition of glucose

solution causes prominent fluorescence decrease of hydrogels. In addition, a decrease in fluorescence intensity was accompanied with increase in glucose concentrations added to the hydrogels. The decrease of fluorescence also shows concentration-dependent behavior, in good agreement with the observation from the microplate reader.

3.5.2. Glucose sensing using PEG-FOA-GO_x hydrogel prepared with 20 mM pH 7.4 sodium phosphate buffer containing 150 mM NaCl (GO_x loading of 10 mg/mL)

Similar glucose detection experiments were also conducted using PEG-FOA-GO_x hydrogel prepared with 20 mM sodium phosphate buffer containing 150 mM NaCl. As previous study also shows that 10 mg/mL GO_x loading also possesses good response to glucose, a lower GO_x loading (10 mg/mL) was used in this hydrogel in order to reduce the cost of GO_x. As shown in Figure 23, one can see that the reduced loading of GO_x does not change the response pattern too much compared to the one using 20 mg/mL, indicating that good glucose response can also be achieved with low GO_x loading (low cost) in the presence of physiological level of NaCl. The corresponding time-depended normalized fluorescence intensity change is presented in Figure 24. The fluorescence images of the hydrogel immediately and after 7 hours addition of different glucose solution were also obtained under UV-light at 365 nm wavelength and presented in Figure 25. Compared the hydrogel without NaCl, this hydrogel's initial fluorescence is slightly

weaker, which may be ascribed to the quench from higher ionic concentration. Similar as previous discussion, a concentration-dependent fluorescence was observed, which matches the fluorescence readings data very well.

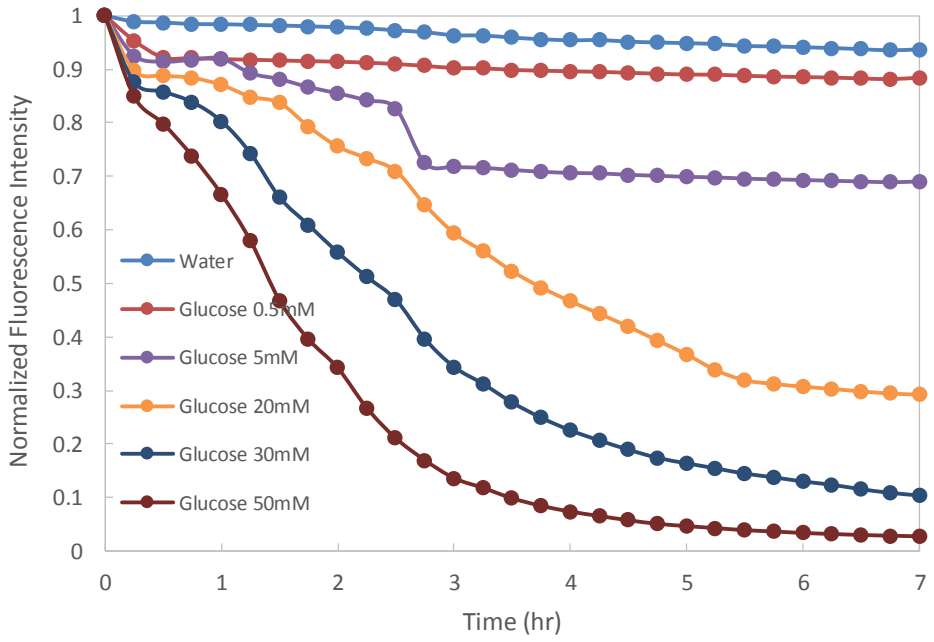


Figure 23: *The time-dependent normalized fluorescence intensity of PEG-FOA-GO_X hydrogels prepared with 20 mM pH 7.4 sodium phosphate buffer containing 150 mM NaCl (GO_X loading=10 mg/mL) upon the addition of glucose solutions with different concentrations.*

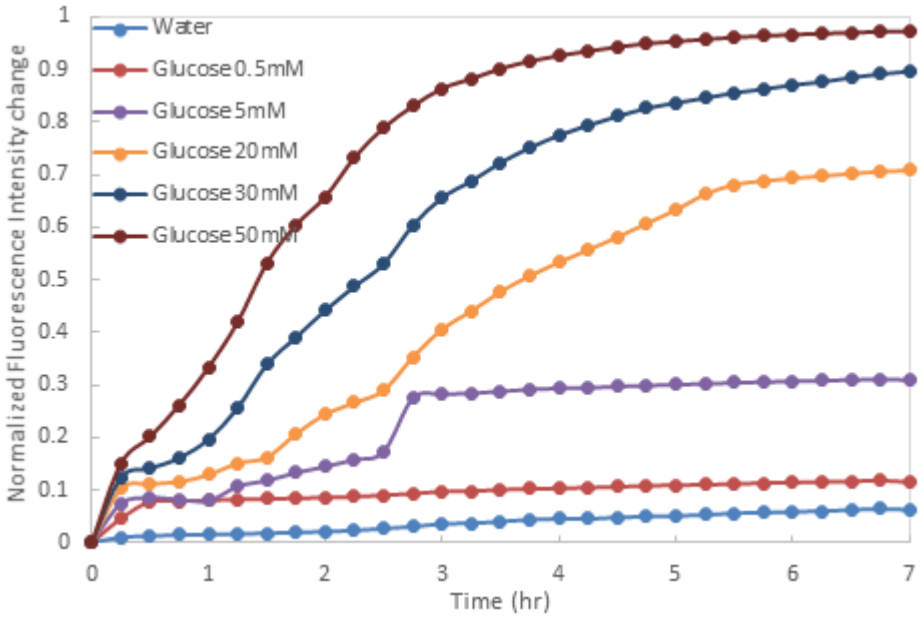


Figure 24: *The time-dependent normalized fluorescence intensity change of PEG-FOA-GO$_X$ hydrogels prepared with 20 mM pH 7.4 sodium phosphate buffer containing 150 mM NaCl (GO$_X$ loading=10 mg/mL) upon the addition of glucose solutions with different concentrations.*

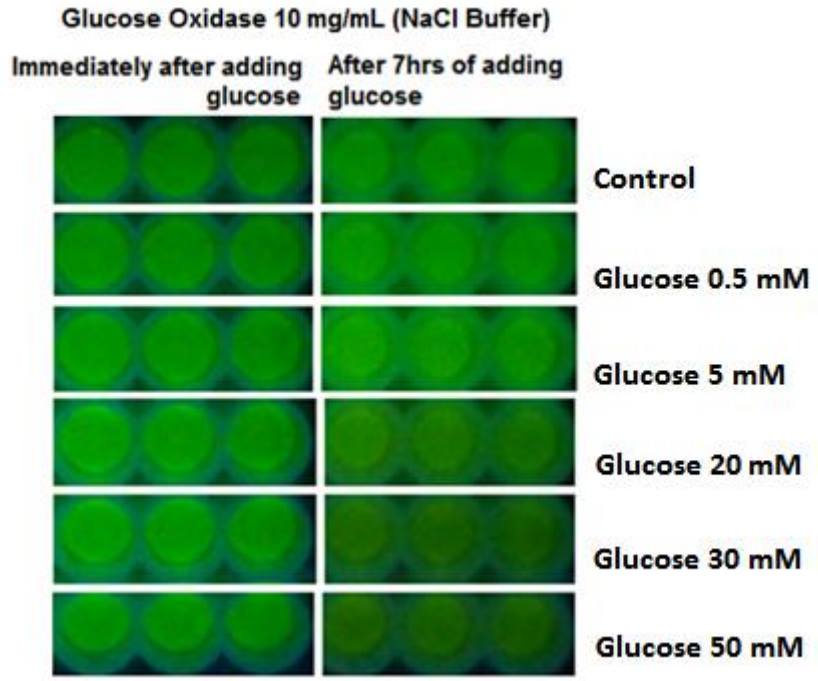


Figure 25: *The fluorescence images of PEG-FOA-GO$_x$ hydrogels prepared using 20 mM pH 7.4 sodium phosphate buffer containing 150 mM NaCl under UV-light at 365 nm immediately and after 7hrs addition of glucose solution with different concentrations.*

3.5.3. Glucose sensing using PEG-FOA-GOx hydrogel fiber (GOx loading of 20 mg/mL)

Inspiring from the high performance of the as-prepared hydrogel in glucose monitoring we have come up with a hydrogel fiber to improvise its response time. To form a hydrogel fiber the same procedure of forming hydrogel gel is followed where fluorescein-o-acrylate (FOA) and polyethylene diacrylate (PEGDA) mixture containing GO$_x$ was polymerized with TEMED and APS as accelerator and

initiator, respectively. 10% SDS solution is used to rinse the tubing first in order to reduce the friction between the fiber and the walls of the tube during extrusion process later. Before the mixture gets polymerized it is thoroughly mixed and loaded into a PVC tube of diameter 1/32 inches with the help of a syringe. Chemical polymerization initiated by APS (initiator) and TEMED (accelerator) which accelerates the formation of free radicals from persulfate catalyzes and polymerization takes place in the tube. The elongating polymer chains are randomly crosslinked by PEGDA (also serving as crosslinker), resulting in a hydrogel fiber in tubing. After the formation of hydrogel, the hydrogel fiber was extruded from the tubing and the as-prepared glucose-responsive fluorescent hydrogel fiber provides an excellent biosensing platform for glucose monitoring.

Figure 26A and 26C explains the similar fluorescence intensity irrespective of forming a hydrogel or hydrogel fiber, when observed under UV-light of 365 nm wavelength.

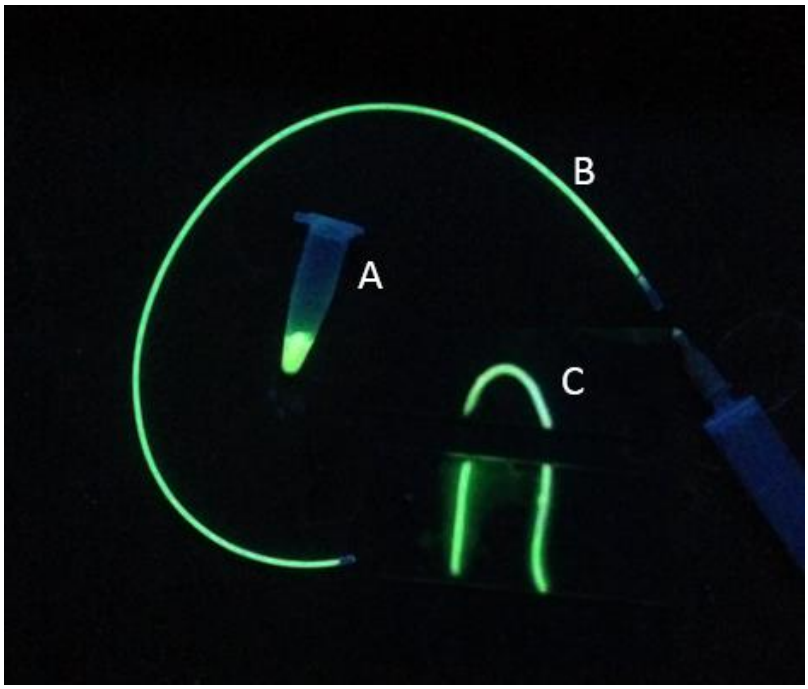


Figure 26. *(A) Hydrogel formed in an eppendrof tube, (B) hydrogel fiber inside PVC tube of inner diameter 1/32 inches, and (C) hydrogel fiber after extrusion from the PVC tube and placed on a glass slide.*

Similar glucose detection experiments were also conducted using PEG-FOA-GO_x hydrogel fiber prepared with 20 mM sodium phosphate. As previous study shows that 10 mg/mL GO_x loading possesses good response to glucose in a hydrogel, similar GO_x loading was used in the prepration of hydrogel fiber. FigureAs shown in Figure 27, three hydrogel fibers are treated in water, 20 mM glucose solution and 100 mM glucose solution, respectively. Within 5 min we could see a clear fluorescence decrease in the hydrogel fiber treated with 100 mM glucose. A similar fluorescence decrease is expected in the hydrogel fiber that is treated with 20 mM glucose, but since the fluorescence change is small when compared to sample

(C), it is not obvious to naked eye. This experiment indicated that hydrogel fiber shows much faster response upon the injection of glucose, which can be attributed to fast diffusion of glucose into hydrogel because of its small diameter.

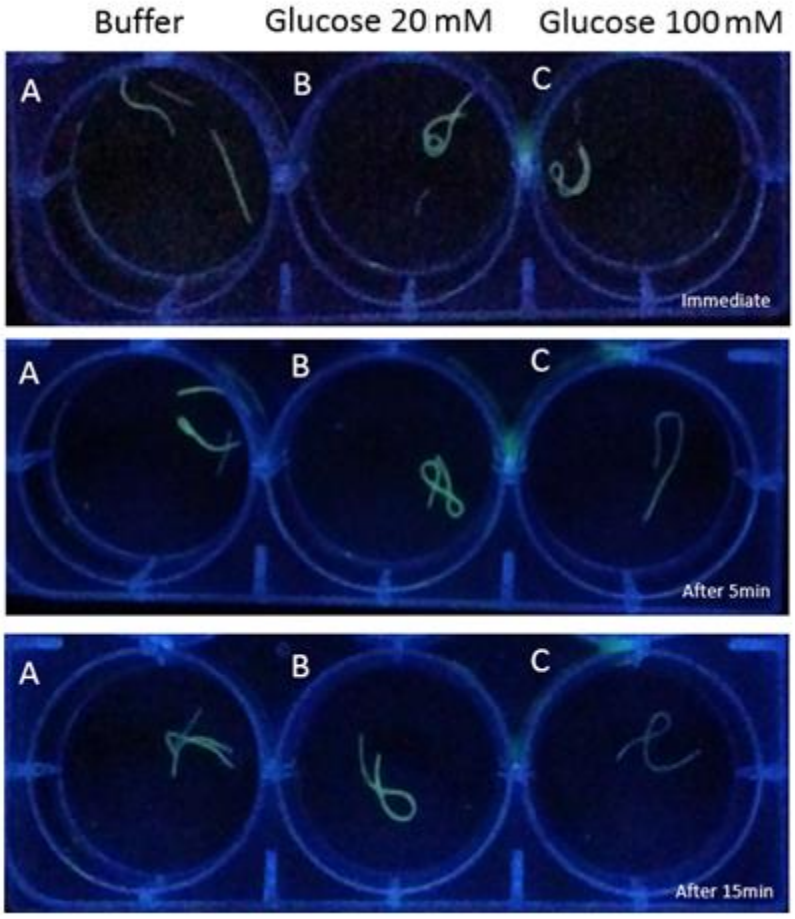


Figure 27: *Glucose detection by FOA-PEG-GO$_x$ hydrogel fiber, sample (A) treated with water, sample (B) treated with 20 mM glucose solution and sample (C) treated with 100 mM glucose solution.*

Chapter 4

Conclusion and Future Direction

4.1 Conclusion

Throughout this thesis, we have explored a facile approach for the synthesis of PEG-FOA hydrogel with simultaneous entrapment of GO_x. Taking the advantages of PEG and strong fluorescence of fluorescein, we used chemical polymerization to develop fluorescence hydrogel using PEG diacrylate and fluorescein-o-acrylate as monomers and PEG diacrylate as crosslinkers. The addition of GO_x into polymerization solution resulted in glucose-responsive fluorescence hydrogel (PEG-FOA-GO_x). As FOA is incorporated into the backbone of hydrogel, the leaching of fluorophore is significantly minimized, endowing the hydrogel stable fluorescence. Two types of hydrogels were prepared using 20 mM pH 7.4 sodium phosphate buffer with or without 150 mM NaCl. The presence of 150 mM NaCl in hydrogel preparation has no significant effect on glucose response pattern. The effect of GO_x loading in hydrogel on biosensor response is also investigated. Then two hydrogels biosensors (different GO_x loading) were applied for glucose detection. As glucose diffuses into the hydrogel, gluconic acid is produced due to GO_x triggered enzymatic reaction. This enzymatic product then reacts with the fluorescein motif in the hydrogel, thus resulting in its fluorescence “turn-off”, which can be measured and correlated with glucose concentration.

This design of the biosensing system is efficient in glucose sensitivity and stability. However, since the hydrogel is prepared in a 96-well plate its thickness is relatively high for which glucose takes time to diffuse into the hydrogel. The delay in diffusing glucose into the hydrogel delays the reaction between glucose and the enzyme, accordingly. Though the observed quenching efficiency is relatively good, the performance can be further improved using fluorescence hydrogel fiber. Due to the small diameter of the hydrogel fiber (~750 μm), glucose can quickly diffuse into hydrogel fiber and thus a faster response towards glucose injection could be observed. As PEG has well-documented biocompatibility, the as-developed glucose-responsive fluorescent hydrogel and hydrogel fiber hold great promise in the development of injectable glucose biosensors for continuous glucose monitoring.

4.2 Future Direction:

As the developed glucose-responsive hydrogel shows good response to glucose, some of future tasks are proposed. First, in vitro continuous glucose sensing can be conducted to further evaluate the sensing performance of the developed PEG-FOA-GO_x hydrogel because glucose concentration in human body continuously changes, thus requiring continuous monitoring. Therefore, a simulated in vitro continuous glucose sensing can be conducted in a PDMS microfluidic system. In this future study, the as-prepared hydrogel fiber will be fixed on glass slide first. Standard soft lithography techniques are then applied to create polydimethylsiloxane (PDMS) microfluidic channels. To complete the device

fabrication the PDMS channel will be put on the top of fixed hydrogel fiber and bonded to the glass, and then assembled with appropriate connectors (inlet and outlet) to form a microfluidic system. Due to the transparency of PDMS and glass, their presence would not affect taking fluorescence images of fluorescent PEG-FOA-GOx hydrogel fiber in microfluidic channel. By regulating the perfusion rate of glucose solution with different concentrations, real-time glucose monitoring can be achieved through the capture of the fluorescent images of hydrogel fiber followed by analysis using ImageJ software. The long-term stability (or lifetime) of the developed glucose-responsive hydrogel will also be investigated by keeping running the experiments at 37 °C. After in vitro study, in vivo study can be further carried out using a rat subcutaneous model in conjunction with the biocompatibility study.

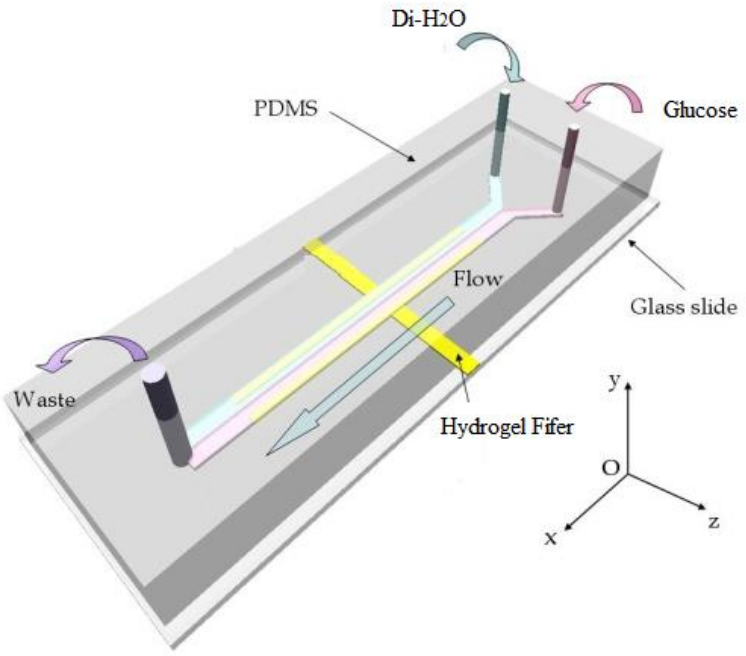


Figure 28: *PDMS microfluidic channel for CGM.*

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