DEVELOPMENT OF A CLICK HYDROGEL PLATFORM FOR CHEMO-OPTICAL GLUCOSE SENSING

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Development of a Click Hydrogel Platform for Chemo-Optical Glucose Sensing

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A need exists for the development of a continuous glucose monitoring system to improve the management and treatment of diabetes mellitus. Click hydrogels are a promising platform for an implantable, continuous glucose monitoring system because of their ease of fabrication, highly specific chemistry, tunable properties, and ability to incorporate sensing moieties.[1,2] Recent research has demonstrated the ability to monitor glucose levels by immobilizing glucose sensing enzymes, specifically glucose oxidase (GOx), within a hydrogel network. When coupled with an oxygen sensitive phosphorescent dye, glucose levels can be quantified optically when interrogated with light[3]. To advance this technology, this project builds on previous work in our lab that demonstrated the ability to create protein functionalized hydrogel microparticles utilizing sequential click reactions. By harnessing this powerful chemistry, the GOx enzyme can be modified with a tetrazine group in aqueous solution and then subsequently added in 8, 4, 2, 1, and 0.5 mg/mL GOx concentrations to hydrogel microparticles. This work will seek to demonstrate the ability of click hydrogel microparticles to serve as a promising chemo-optical glucose-sensing platform for continuous glucose level monitoring.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Motivation

Monitoring blood glucose levels is of critical importance for patients with diabetes mellitus. Current glucose sensing technologies rely heavily on methods requiring a finger prick, such as the Glucometer, which tests the glucose levels in a drop of the patient's blood[4]. While effective, current glucose monitors can cause discomfort, patient compliance can pose a challenge, and the technologies currently available are not capable of continuous glucose level monitoring. Recent research has been devoted to using glucose sensing enzymes, specifically the glucose oxidase (GOx) enzyme, immobilized within a hydrogel scaffold to develop a stable and implantable glucose sensor[3]. Enzyme based glucose sensors show promise for the development of glucose biosensors due to their high substrate specificity, high availability, and their stability in physiological conditions. When coupled with an oxygen sensitive phosphorescent dye, enzyme-based glucose sensors can be used to optically measure glucose levels *in vivo* and even monitor glucose levels continuously[3].

Hydrogels are polymeric materials capable of absorbing large amounts of water, and are widely used for biomedical applications because of their similarity to soft tissue in the body. Given their hydrophilic nature and blank slate foundation, hydrogels lend themselves as a valuable tool for the specific incorporation of proteins, binding sites, and drug delivery in a variety of applications in diagnostics and therapeutics[1]. Click hydrogels have recently emerged as a powerful new tool due to their high reaction specificity, ease of fabrication, tunability after

synthesis, and potential for bio-orthogonality[2]. Click chemistry encompasses a variety of reactions, including photo-initiated thiol-ene reactions[5], thiol Michael addition reactions, tetrazine based inverse-electron-demand Diels Alder reactions, and copper catalyzed or strain promoted azide-alkyne cycloaddition reactions. For biomedical purposes, and specifically for this work, thiol-ene reactions and tetrazine based click reactions were chosen due to their metal-free initiation[6,7] and ability to utilize norbornene functionalities in a step-wise manner.

A key aspect of enzyme based sensing is control over the delivery of the analyte to the enzyme. The GOx enzyme consumes its substrates, glucose and oxygen, in a 1:1 molar ratio. For an implantable sensor, possessing the ability to control the diffusion of glucose and oxygen to the sensing enzyme is crucial for sensor reliability and performance. While optical sensing technology has improved, factors such as protein encapsulation, or bio-fouling, and host rejection can cause the rate of substrate diffusion into the sensor to vary, requiring frequent sensor recalibration[8]. To mitigate this problem and develop a sensing system capable of both continuous glucose monitoring and controlling substrate diffusion, a core shell design was chosen for this work.

Considering the advantages detailed above, click hydrogels show promise for the development of an enzyme based, chemo-optical glucose monitoring system.

Objective

The objective of this work is to use sequential click reactions to form hydrogel microparticles that can be utilized as a platform for the development of a chemo-optical glucose monitoring

system. Specifically, sequential thiol-ene and tetrazine-norbornene click reactions will be used to form microparticles and then conjugate the GOx enzyme to the microparticles in a simple, two-step process. A phosphorescent dye will also be embedded within the hydrogel network so that glucose levels can be measured optically[3]. The rate of substrate diffusion into the sensor will be controlled as well by implementing a core shell design for the microparticles.

CHAPTER II

METHODS

Fabrication of the click hydrogel platform

Due to their high surface area to volume ratio, poly(ethylene glycol)-norbornene (PEG-NB) microparticles were chosen as the hydrogel platform. Fabrication of the microparticles is accomplished by the suspension of a 10 wt% PEG-NB solution within a 40 wt% dextran solution. Due to the property of polymer immiscibility, the 10 wt% PEG-NB phase containing a 1:0.75 molar ratio of PEG-NB to the thiols (SH) of the cross-linker, dithiothreitol (DTT), and 2 mM photo-initiator lithium acylphosphanate (LAP) remains separate from the 40 wt% dextran and 4.55 mM LAP phase until UV exposure for 5 minutes under 10 mW/cm^2 at 365 nm to initiate crosslinking. The microparticles were then isolated through a series of washes followed by centrifugation before suspension in a PBS solution for later use. A visual for the microparticle preparation is given below in **Figure 1**.

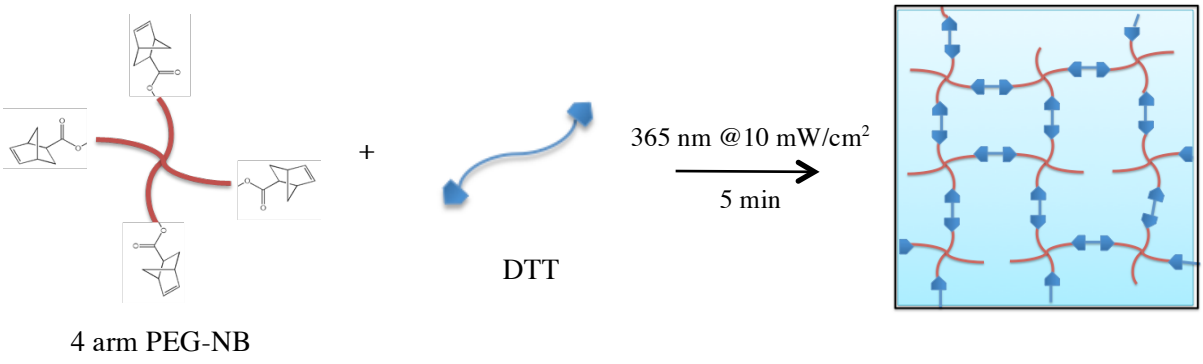

Figure 1. Representation of the thiol-ene polymerization used to create the PEG-NB microparticle network. The thiol containing DTT crosslinker is added to the PEG-NB in an 0.75:1 SH:NB ratio to stoichiometrically control the free norbornene groups in the hydrogel for subsequent click reactions.

Tetrazine-GOx modification

Using a protocol previously established in our lab, the GOx enzyme was prepared for PEG-NB microparticle functionalization by the addition of 10 molar equivalents of a tetrazine (Tz)-NHS ester dissolved in DMSO to GOx in solution and incubated for one hour at room temperature. The solution of tetrazine modified GOx (Tz-GOx) was then diluted to 8, 4, 2, 1, and 0.5 mg/mL GOx concentrations for immobilization within the PEG-NB microparticles. Utilizing the free norbornene groups ensured by the 1:0.75 molar ratio of NB to SH used to create the microparticles, the Tz-GOx solutions were then added to pelleted PEG-NB microparticles and incubated for one hour to allow tetrazine-norbornene click chemistry to tether the Tz-GOx groups to the microparticles, as shown below in **Figure 2**. Following conjugation, a series of PBS washes and centrifugation were performed followed by an overnight suspension in a PBS sink to ensure removal of unconjugated GOx from the microparticles.

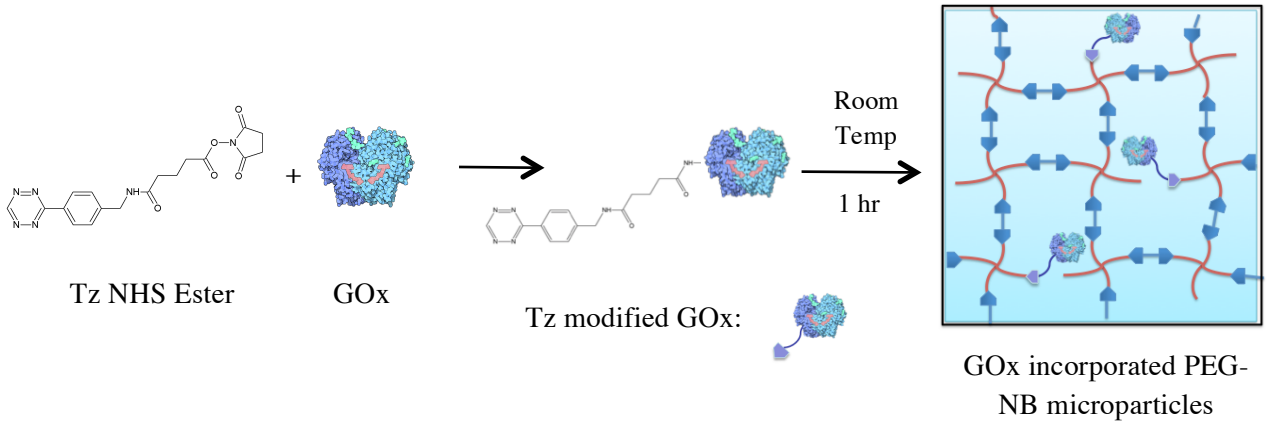

Figure 2. Schematic illustrating the modification of the GOx enzyme with tetrazine by the addition of 10 molar equivalents of a tetrazine NHS ester to GOx in aqueous solution. The Tz-GOx solution was then incubated with PEG-NB microparticles in 8, 4, 2, 1, and 0.5 mg/mL GOx concentration in aqueous conditions at room temperature for one hour. Because of the stoichiometrically controlled free norbornenes in the network, GOx could be covalently incorporated into the microparticle network via tetrazine norbornene click chemistry.

The microparticles were equilibrated in a sodium acetate (NaOAc) buffer for one hour before enzymatic activity of the immobilized GOx was confirmed using an adapted protocol[9], in which the change in absorbance values over time at 490 nm were recorded using a Tecan Infinite 200 Pro Series Plate Reader. The reaction solution used consisted of: 1.67 wt% D-glucose, 0.007 mg/mL horseradish peroxidase (HRP), and 1.68 μM o-dianisidine dye in sodium acetate buffer. In this assay, the bioactivity of the enzyme is measured based on the production of hydrogen peroxide as GOx consumes its substrates, glucose and oxygen. The hydrogen peroxide produced reacts with HRP and oxidizes the o-dianisidine dye to produce the color change measured by the plate reader.

Building a chemo-optical glucose sensor

Incorporation of a phosphorescent dye into the PEG-NB microparticles is a key component in the development of a glucose monitoring system. The sensing dye functions as the chemo-optical portion of the glucose monitor, enabling phosphorescent intensities to be measured optically and then correlated with glucose concentrations during *in vitro* testing. For this work, a palladium(Pd)(II) pentafluorophenyl porphyrin dye, shown below in **Figure 3**, was selected as the oxygen sensing dye due to its availability, prior use, and the high quantum yield it possesses[10].

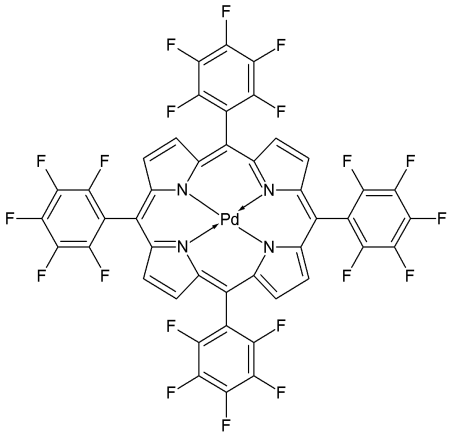

Figure 3. Chemical structure of the Pd(II) pentafluorophenyl porphyrin dye chosen for the chemo-optical glucose sensing system.

In physiological conditions, the dye molecule is quenched by oxygen, and the concentration of oxygen surrounding the dye molecule is reflected in the phosphorescent emission intensity of the dye when interrogated with light. This phosphorescent lifetime provides a means of measuring oxygen consumption, and when coupled with the GOx enzyme, the consumption of glucose as well, to then serve as a real time measurement of the glucose concentrations in the environment. The dye however is hydrophobic, posing a challenge to its incorporation within a hydrogel network. To overcome this obstacle, a protocol by *Koren et al*[10] was adapted to perform a nucleophilic substitution of the fluorine atoms surrounding the Pd(II) core of the dye for poly(ethylene glycol)(PEG)-di-thiol (SH-PEG-SH, Mw = 3400 Da) groups. This substitution not only increases the hydrophilicity of the dye, but also allows incorporation of the dye within the click hydrogel network in a manner amenable to the current microparticle synthesis using the sequential thiol-ene and tetrazine-norbornene click reactions. Using a 30:1 molar ratio of di-thiol to Pd(II) porphyrin, the PEG-di-SH, porphyrin, and 5 molar equivalents of triethylamine were dissolved in dimethyl formamide (DMF) and stirred at 75°C for 2 hours. The product was then

precipitated using a 10-fold excess of diethyl ether, filtered, and then dried before dissolution in DI water to 10 wt% PEG concentration for subsequent incorporation into the PEG-NB microparticles.

Replacing the PEG-NB microparticle cross-linker, DTT, the PEG-Pd(II) porphyrin complex was added to the PEG-NB microparticle solution at a 0.75:1 SH:NB ratio and exposed to UV light to initiate crosslinking.

Synthesis of the oxygen sensing phosphorescent dye

In order to have sufficient pentafluorophenyl porphyrin dye for testing the necessary parameters for incorporating the PEG-modified dye into the PEG-NB hydrogel microparticle network, synthesis of the tetrakis pentafluorophenyl Pd(II) porphyrin dye was attempted based on an adapted protocol by *Lindsey et* al[12]. In addition to reduced cost compared to commercially available porphyrin dye, synthesis of the dye also ensures greater control over the functional groups surrounding the dye molecule for subsequent incorporation into the click hydrogel microparticle platform. To synthesize the un-metalated dye precursor, pyrrole (10 mmol), and benzaldehyde (10 mmol) were dissolved in 1 liter of dichloromethane (DCM) in a large round bottom flask and stirred at room temperature for several minutes before the catalyst, boron trifluoride diethyl etherate (3.3 mmol), was added. This mixture was covered and stirred under nitrogen for one hour before oxidizing with tetrachloro-1,4-benzoquinone (7.5 mmol). This reaction was then allowed to proceed for 3 hours while stirring at 50°C with a refluxing column attached to the top of the flask. The product was then removed from the oil bath and rotavapped to remove DCM, and then stored at -20°C before column filtration. In order to purify the dye

precursor in preparation for Pd(II) metalation, the product was dissolved in DCM and passed through a silica column several times until the solution changed color from a dark gray/black to purple. The precursor was then precipitated with the addition of methanol, and then the solution was passed through a paper filter to collect the purified dye precursor. This precursor was then dried, dissolved in DMF with 3 molar equivalents of Pd(II)Cl, and then allowed to react overnight at 120°C with a refluxing column placed over the flask to metalate the core of the dye molecule with Pd(II). The solution was then removed from the oil bath and the DMF was pulled off using the rotavapp. The dried dye product was dissolved in DCM before passing through a silica column to purify. Following purification and collection, the dye solution was poured into a flask and attached to the rotavapp again to pull off the DCM. Methanol was then added in excess to precipitate the final product and the mixture was poured over a paper filter to collect the Pd(II) metalated porphyrin dye.

Characterization was performed using ^{1}H-NMR spectroscopy. Identification of incomplete metalation was noted and the overnight metalation reaction was performed again by adding 3 molar equivalents of Pd(II)Cl and stirring the solution in DMF overnight at 130°C. The reaction was then removed from the oil bath and the DMF was pulled off before dissolution of the product in DCM, filtration through a column of silica, and subsequent collection and solvent removal. Methanol was added to the dye product in excess and the flask was cooled to -20°C to aid in precipitation.

Following a second incomplete metalation attempt of the dye precursor molecule, the reaction was attempted a third and final time using a modified protocol by *Wai-Pong To et al*[14] in

benzonitrile. Benzonitrile was chosen as the solvent for the final metalation attempt because it binds to Pd(II) in an intermediate step to assist complete insertion of the Pd(II) into the core of the porphyrin dye molecule. The unmetalated dye precursor and 3 molar equivalents of Pd(II)Cl were dissolved in benzonitrile and the reaction was refluxed under argon for 24 hours. The reaction mixture was then removed from the oil bath and allowed to cool to room temperature before removal of the benzonitrile and dissolution in DCM followed by silica column filtration in order to purify the product and remove excess Pd(II)Cl. The collected product was then added dropwise to methanol and cooled to -20°C to precipitate the metalated dye product.

Following several unsuccessful attempts to synthesize the Pd(II) pentafluorophenyl porphyrin dye for PEG-modification of the dye molecule and subsequent PEG-NB hydrogel network incorporation, a second nucleophilic substitution reaction was performed on commercially available Pd(II) pentafluorophenyl porphyrin dye, shown below in **Figure 4**. For this reaction, thiol terminated PEG chains were chosen to substitute the *para* position fluorine atoms surrounding the dye molecule to allow incorporation of the oxygen sensing dye into the hydrogel platform of the glucose sensor in a manner amenable to the sequential click synthesis currently used.

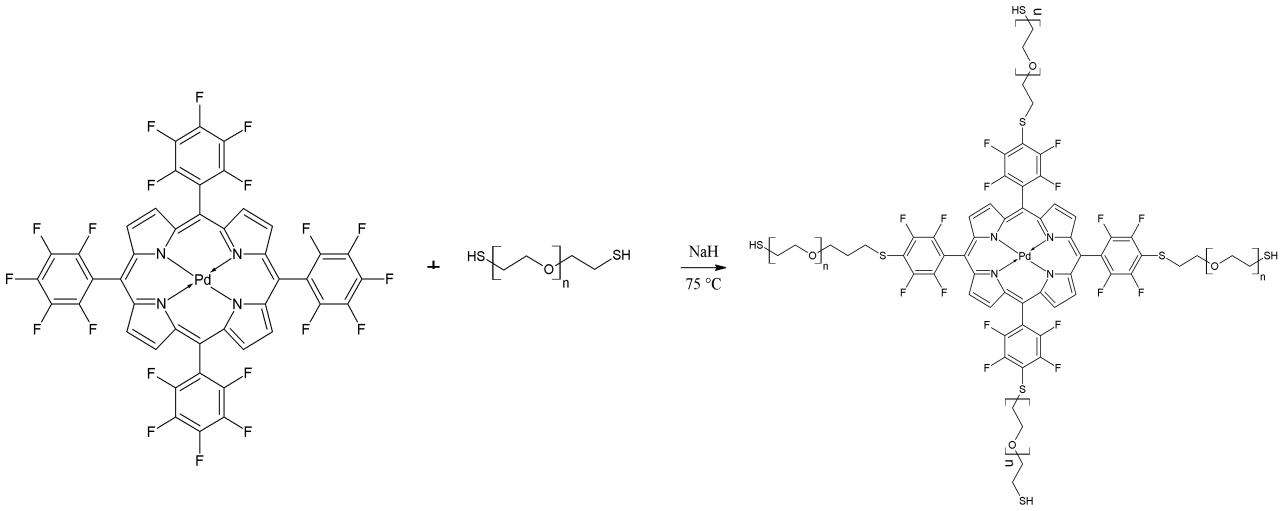

Figure 4. Schematic detailing the nucleophilic substitution of the *para* position fluorine atoms surrounding the Pd(II) pentafluorophenyl porphyrin dye molecule for thiol terminated PEG chains. The reaction was performed in DMF in the presence of a dry solvent, NaH, to act as the deprotonating agent.

A round bottom flask was purged with nitrogen before 0.4 g of NaH was added to 8 mL of DMF in the base of the flask. Separately, 20 molar equivalents, 1 g, of PEG-di-SH and 1 molar equivalent, 15 mg, of Pd(II) pentafluorophenyl porphyrin dye were dissolved in 20 mL, and 5 mL of DMF respectively. While stirring under nitrogen, the PEG-di-SH was added dropwise through a syringe into the NaH and DMF mixture. After completing the addition of the PEG-di-SH, the mixture was allowed to stir for 20 minutes in order to ensure complete deprotonation of the thiol terminated PEG chains, which was apparent when hydrogen gas evolution from the vessel ceased. The Pd(II) pentafluorophenyl porphyrin dye was then added dropwise to the mixture while stirring under nitrogen. After stirring several minutes, the mixture was covered and heated to 75°C. The reaction was permitted to proceed for 5 hours before cooling to room temperature and passing through a paper filter to remove sodium fluoride salt. The product was then collected and added to a 10-fold excess of diethyl ether to precipitate and collected via

paper filtration again before rinsing twice in diethyl ether using centrifugation. ^{1}H-NMR spectroscopy samples were prepared of the PEG substituted Pd(II) pentafluorophenyl porphyrin dye and Pd(II) pentafluorophenyl porphyrin dye (each containing ~0.15 mM porphyrin) in DMSO d6 for analysis.

Synthesis of the Tz-PEG-Tz crosslinker

While thiol-ene polymerization was successful in creating the PEG-NB hydrogel microparticle platform for functionalization with GOx, incorporation of the phosphorescent dye in a photo-initiated system was found to prohibit network formation due to an interaction of the UV light source with the porphyrin dye. In order to address the challenge of utilizing a non photo-initiated system for microparticle fabrication, tetrazine-norbornene chemistry was chosen as an alternative means of creating the PEG-NB hydrogel microparticle network. In order to replace the DTT crosslinker in the former PEG-NB microparticle system, a Tz-PEG-Tz monomer was synthesized based on a modified protocol taken from *Alge et al*[1] and *Kirschner et al*[15] from PEG-bisamine and a tetrazine carboxylic acid (Tz-COOH). By removing the requirement of a photo-initiated system for microparticle formation, incorporation of the phosphorescent oxygen sensing dye, Pd(II) pentafluorophenyl porphyrin, would be possible for future work in building the chemo-optical glucose sensor. The substitution of the DTT for Tz-PEG-Tz as the network crosslinker would also allow the mechanism for GOx incorporation into the PEG-NB hydrogel network to remain the same, simply altering the 0.75:1 SH:NB ratio used in the DTT crosslinking system for a 0.75:1 Tz:NB ratio in the Tz-PEG-Tz crosslinking system, leaving 25% of the norbornene moieties free for functionalization with GOx.

As shown below in **Figure 5**, to synthesize the Tz-PEG-Tz monomer, 0.502 g of PEG-bisamine (Mw = 3400 Da, ~0.3 mmole NH_2) was added to an argon purged scintillation vial, and dissolved in 3 mL of DMF along with 129 μL of DIEA (2.5 equiv.). This mixture was allowed to stir at room temperature for 15 minutes to ensure dissolution of the PEG-bisamine. Separately, a second scintillation vial was purged with argon and to it, 0.225 g of Tz-COOH (2.5 equiv. with respect to NH_2) and 0.280 g HBTU (2.5 equiv. with respect to NH_2) were dissolved in 2 mL of DMF and left to stir 5 minutes at room temperature. Upon ensuring that both mixtures were completely dissolved, the Tz-COOH and HBTU mixture was added to the PEG-bisamine and DIEA mixture and covered with a blanket of argon before capping and leaving to react while stirring at room temperature for 5 hours.

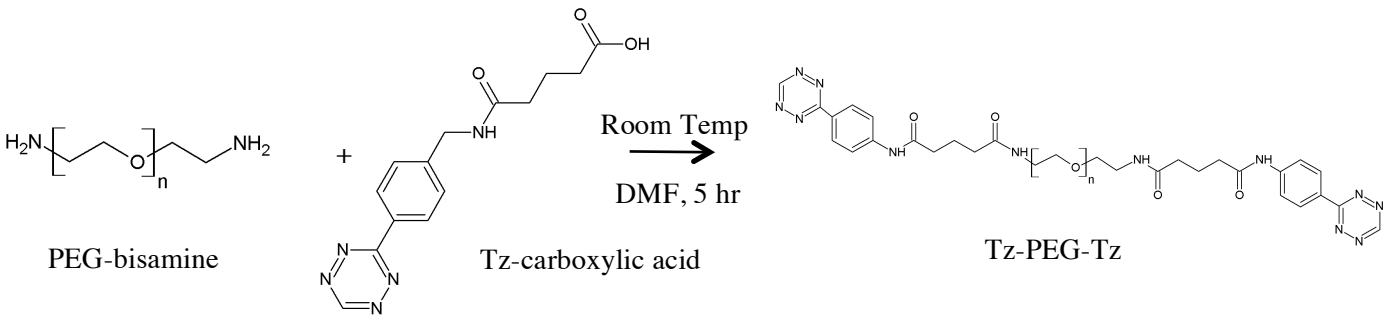

Figure 5. Synthesis of Tz-PEG-Tz was performed through the addition of 2.5 equivalents of Tz-COOH with respect to the amine groups of PEG-bisamine. The reaction was carried out at room temperature under a blanket of argon.

The product was precipitated by the addition of 80 mL of cold diethyl ether, and the mixture was then divided into two 50 mL centrifuge tubes and rinsed 3x via centrifugation before drying. The dried product was then dissolved in DI water and added to 1 kDa dialysis tubing to purify the final Tz-PEG-Tz product. The dialysis tubing was submerged in 4 L of DI water and left to

stir for 48 hours, with fresh DI water replaced after 24 hours. The dialyzed Tz-PEG-Tz product was then poured directly from the dialysis tubing into two 50 mL centrifuge tubes and then frozen at -80°C before lyophilization for 48 hours and ^{1}H-NMR spectroscopy.

CHAPTER III

RESULTS

GOx functionalized click hydrogel microparticle platform

Click hydrogel PEG-NB microparticles were successfully fabricated by suspension of an aqueous solution containing PEG-NB, crosslinker DTT, and photoinitiator LAP within a dextran phase also containing the photoinitiator LAP. Exposure to UV initiated crosslinking of the hydrogel microparticle network and the microparticles were then isolated and suspended in a solution of PBS in preparation for GOx enzymatic functionalization.

The GOx enzyme was prepared for microparticle functionalization as detailed above, by modification with a tetrazine-NHS ester in aqueous solution using 10 molar equivalents of the Tz-NHS ester. The solutions of Tz-GOx were then diluted to 8, 4, 2, 1, and 0.5 mg/mL GOx concentrations and pipetted over pelleted PEG-NB microparticles to allow for tetrazine-norbornene click chemistry to tether the GOx enzyme to the microparticles. Dose dependent GOx activity was confirmed using UV absorption scans of the microparticles suspended in a solution of glucose, horseradish peroxidase to react with the hydrogen peroxide produced as GOx consumes glucose and oxygen, and a dye, which is oxidized to produce the color change measured by the UV plate reader. This color change is measured as the enzymatic bioactivity of the GOx enzyme and the results of the complete study are displayed below in **Figure 6**.

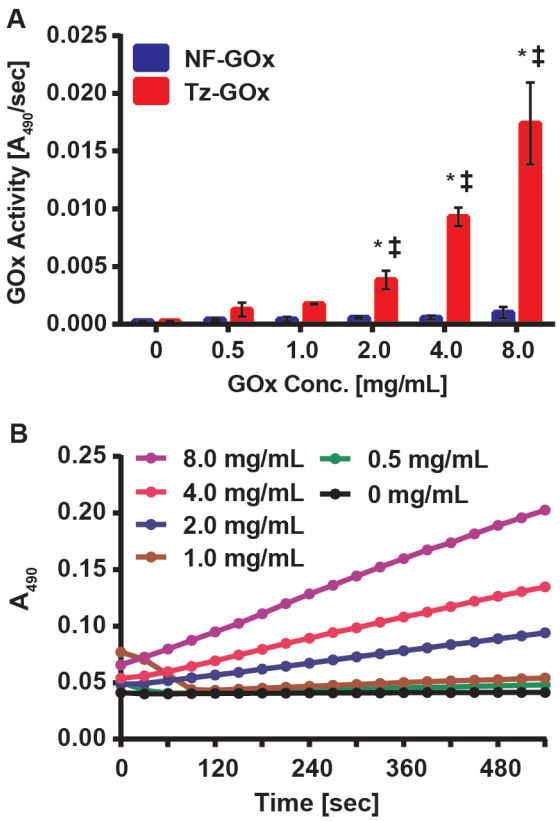

Figure 6. Dose dependent GOx bioactivity demonstrated after PEG-NB microparticle functionalization.[13] (A) Slopes of the UV absorption scans demonstrate the dose dependent bioactivity of the GOx functionalized microparticles: Tz-GOx. The NF-GOx groups consist of PEG-NB microparticles incubated with equivalent concentrations of non-tetrazine modified GOx. Data was analyzed by two-way ANOVA followed by post hoc comparisons (Tukey's method). The symbols * and ‡ indicate significant differences between Tz-GOx and NF-GOx and between Tz-GOx concentrations, respectively ($p < 0.05$). (B) Increasing hydrogen peroxide formation as the Tz-GOx microparticles consume glucose in solution before activity begins to plateau at 8 minutes.

As demonstrated above in **Figure 6** by the bioactivity of the Tz-GOx group, the GOx enzyme was successfully conjugated to the PEG-NB hydrogel microparticles in a dose dependent manner utilizing tetrazine-norbornene click chemistry. The NF-GOx group consisted of equal amounts of PEG-NB microparticles allowed to incubate with non-tetrazine modified GOx at the same enzyme concentrations as used in the Tz-GOx groups: 8, 4, 2, 1, and 0.5 mg/mL. These microparticles were rinsed with PBS and equilibrated in sodium acetate buffer in the same

manner as the Tz-GOx microparticles in order to ensure that the bioactivity of the Tz-GOx functionalized PEG-NB microparticles was a result of dose dependent enzymatic conjugation. Establishing PEG-NB microparticles as a tunable platform for the conjugation of biomolecules using sequential click reactions was demonstrated. The GOx enzyme was successfully conjugated to the microparticles in a dose dependent manner, and shows promise for the development of a continuous glucose sensing platform.

Building a chemo-optical glucose sensor

Following the first attempted nucleophilic substitution of the fluorine atoms surrounding the commercially available Pd(II) porphyrin dye molecule for PEG-di-thiol chains, successful cross-linking of the PEG-NB hydrogel microparticles with PEG-thiol functionalized Pd(II) porphyrin dye was not achieved. A color change of the gel solution indicated that an interaction of the photo-initiator, LAP, with the porphyrin dye damaged the dye molecule and prohibited the photo-initiated polymerization of the network. In order to reduce costs in further scouting, synthesis of the oxygen sensing phosphorescent dye was attempted. In addition to the benefit of reduced cost, synthesis of the dye permits greater control of the functional groups surrounding the dye molecule for potential modification and subsequent incorporation within the click hydrogel network.

The Pd(II) porphyrin dye was synthesized according to an adapted protocol by *Lindsey et al*[12], as detailed above. The dye precursor was successfully synthesized and purified, and characterization was performed following the first attempt of Pd(II) insertion into the core of the dye molecule. ^{1}H-NMR results indicated that the metalation process was incomplete so the

reaction was performed again at an increased temperature and both ^{1}H-NMR and Mass Spectroscopy were utilized to assess Pd(II) metalation. The final dye product was then passed through a column of silica again and an attempted precipitation was repeated with the addition of excess methanol but the product failed to precipitate from solution. ^{1}H-NMR spectroscopy was performed again and the results indicated that the metalation reaction was still not complete so a third metalation attempt was performed according to an adapted protocol by *Wai-Pong To et al*[14], using benzonitrile as the solvent for the Pd(II) insertion. Following the reaction, the mixture was filtered to remove excess Pd(II)Cl and added dropwise to methanol to assist precipitation but the metalated porphyrin dye product could not be successfully precipitated in methanol, indicating that an error occurred in the formation of the dye product. Given the results of the synthesis attempts, the porphyrin dye synthesis was set aside for future investigation in order to return to the project objective of building a chemo-optical glucose sensor built on a hydrogel platform.

To return to the design challenge of incorporating the hydrophobic porphyrin dye into the click hydrogel microparticle platform, a nucleophilic substitution of the fluorine atoms for thiol terminated PEG chains was performed again using commercially available Pd(II) pentafluorophenyl porphyrin dye, however for this substitution, only the *para* position fluorine groups were targeted. The PEG-di-SH chains were deprotonated in preparation for the substitution through dropwise addition of the PEG solution into NaH and DMF while stirring under nitrogen. After ensuring complete deprotonation, which was apparent after hydrogen gas evolution from the mixture ceased, the Pd(II) porphyrin dye was added dropwise to the mixture while stirring and after several minutes, the reaction was covered and heated to 75°C. Throughout the substitution reaction, the mixture changed color from dark green to brown/red, to

brown/green, and then finally to brown/red with green precipitate at the base of the reaction flask. The mixture was then poured over a paper filter to remove sodium fluoride salt from the product. The reaction mixture containing the dye product was then added to a 10-fold excess of diethyl ether to precipitate the modified dye molecule and then filtered and washed twice with diethyl ether via centrifugation.

^{1}H-NMR results and further manipulation of the PEG-di-thiol substituted porphyrin dye indicated that PEG network formation had occurred, with the functionalized porphyrin operating as the crosslinker in the network. Given the challenges posed in both the synthesis of the Pd(II) porphyrin oxygen sensing dye and the photodamage of the porphyrin dye noted earlier which prevented photopolymerization of the PEG-NB hydrogel network, attention was focused to the design challenge of altering the PEG-NB hydrogel microparticle platform to be a non photo-initiated system.

Synthesis of the Tz-PEG-Tz crosslinker

In considering alternative crosslinking mechanisms for click formation of the PEG-NB hydrogel microparticle platform, focus was turned to tetrazine-norbornene click chemistry because of the success of the mechanism in tethering the GOx enzyme to the PEG-NB microparticle network. One of the advantages of click chemistry it its versatility and the building block style of polymer network formation. To replace the DTT crosslinker used in the previous hydrogel microparticle network system, a di-functionalized tetrazine PEG macromer was chosen. This simple substitution in the hydrogel microparticle network still allowed for the 4 arm PEG-NB to remain as the backbone in the hydrogel network, and the switch simplified reaction conditions given the

spontaneous nature of the tetrazine-norbornene click reaction that requires no external initiation system. The tetrazine based crosslinker was synthesized from PEG-bisamine and a tetrazine carboxylic acid based an adapted protocol taken from *Alge et al*[1] and *Kirschner et al*[15], as detailed above. The final product was isolated, dialyzed to purify, and then lyophilized before ^{1}H-NMR spectroscopy was performed to assess the successful formation of the PEG macromer. Functionalization of the PEG with tetrazine was determined to be ~95% efficient and the product yield was ~70%, comparable to the results reported in the literature[1].

Future directions

Future work will be devoted to verifying and refining the techniques for creating PEG-NB hydrogel microparticles using the newly synthesized Tz-PEG-Tz crosslinker. Replacement of the DTT for Tz-PEG-Tz as the network crosslinker not only simplifies the reaction conditions for microparticle fabrication, but it also allows the tethering of the GOx enzyme to the microparticle network to remain the same through stoichiometric control of the norbornene groups utilized to form the hydrogel network initially. Electrospraying will be investigated as an alternative to the dextran phase suspension polymerization of the microparticles, given its superior control over microparticle size and its capability of rapidly generating monodisperse hydrogel microparticles.

Future work will also be devoted to the investigation of Pd(II) porphyrin dye encapsulation within the hydrogel microparticle based glucose sensing system. Recent work by *Su et al*[16] has demonstrated the ability to encapsulate a platinum(Pt)(II) porphyrin oxygen sensing dye in micelles, which could overcome the challenge of incorporating the hydrophobic dye within a hydrogel material. The micelles were generated by utilizing a poly(ε-caprolactone)(PCL)-block-

poly(ethylene glycol)(PEG) copolymer, with the dye residing within the PCL hydrophobic core and the PEG serving as the external surface of the micelle. The micelles demonstrated stability in aqueous conditions even with dilution, and excellent retention of the phosphorescent dye was observed over the course of 48 hours. The micelle encapsulated porphyrin dye even demonstrated greater quantum yield than free dye in organic solution when interrogated with light. Phosphorescent emission of the dye incorporated within the micelles showed excellent response to increased oxygen concentration in the environment, with a 95% change in intensity within 1 minute of the addition of oxygen. This response time is comparable to that of porphyrin dye incorporated within poly(hydroxyethyl methacrylate) (pHEMA) hydrogel matrices, a material platform commonly used for oxygen sensing applications. Recognition of a deoxygenated environment was slightly more delayed, about 6-7 minutes, but the reaction times from the study indicate that the micellular encapsulation of the porphyrin dye is a viable candidate for a novel oxygen sensing material platform. In one final study, GOx was added in a 10 mg/mL concentration to a solution of glucose that also contained the porphyrin dye incorporated micelles. Response time of the phosphorescent dye within the micelles to the consumption of oxygen by GOx was less than one minute. While this time is slower than that observed by porphyrin encapsulated within other polymer bead systems, the delivery of a hydrophobic dye within a micelle possessing a hydrophilic shell shows promise for the development of a chemo-optical glucose monitoring system given the stability of the micellular structure, the permeability of the micelle to molecular oxygen, and the PEG-ylated external surface that could easily be modified to possess click compatible moieties.

Following the successful fabrication of a click hydrogel platform containing both GOx and an oxygen sensing phosphorescent dye, an adapted protocol for interfacial polymerization[11] will be used to achieve a core-shell design for the glucose monitoring system that will better control the rate of analyte delivery to the glucose sensing enzyme. A poly(hydroxyethyl methacrylate) (pHEMA) shell will be formed around the GOx functionalized hydrogel microparticles via radical chain polymerization by taking advantage of the GOx present on the surface. The GOx functionalized microparticles will be suspended in a coating solution of 15 wt% pHEMA, a 24.5:1 molar ratio of HEMA to the crosslinker tetraethylene glycol diacrylate (TEGDA), 0.1 M D-Glucose, 12.5 mM iron (II) chloride (Fe(II)Cl), and 0.02 wt% fluorescein-o-acrylate for imaging purposes. In the presence of Fe(II)Cl, the peroxide produced as GOx consumes glucose will be converted into hydroxyl radicals, which will initiate radical polymerization at the interface of the microparticles and the coating solution. Polymerization will be arrested by the addition of a 6 mM ethylenediaminetetraacetic acid (EDTA) solution, and shell thickness can be controlled by the time allowed for the reaction to proceed before the addition of the EDTA solution. Finally, the core shell microparticles will then be isolated by a series of PBS washes and centrifugation followed by fluorescence microscopy to confirm the successful formation of the pHEMA shell.

After successful shell formation around the GOx-functionalized and dye-incorporated hydrogel platform is confirmed, glucose diffusion analysis and phosphorescence testing will be performed according to a protocol by *Roberts et al*[8] to determine the sensor's ability to detect analyte levels *in vitro*.

CHAPTER IV

CONCLUSION

In conclusion, click hydrogel microparticles were successfully demonstrated to be a promising platform for the development of an enzyme based, chemo-optical glucose monitoring system. Utilizing a 4 arm PEG-NB molecule, sequential thiol-ene and tetrazine-norbornene click reactions were used to create a hydrogel microparticle matrix in a stoichiometrically controlled fashion, leaving 25% of the norbornenes in the network free for subsequent incorporation of tetrazine modified GOx at 8, 4, 2, 1, and 0.5 mg/mL GOx concentrations via tetrazine-norbornene click chemistry. The enzyme functionalized microparticles also demonstrated dose dependent bioactivity, further showcasing the tunability of the system for the development of an implantable glucose monitor.

The click hydrogel platform chosen for the glucose sensor is not only superior in its high reaction speed and specificity, but click chemistry allows for versatility of network components such as crosslinkers without significant impact to the overall polymer matrix. Because of this, even with the indication that future work will require a transition from the photo-initiated thiol-ene polymerization formerly used to create the microparticle platform, other click reactions are possible with the 4 arm PEG-NB backbone remaining the same, such as Michael type additions, or inverse-electron-demand Diels-Alder reactions such as the tetrazine-norbornene reaction. A tetrazine based crosslinker was successfully synthesized for such purposes and shows promise for the development of the PEG-NB microparticle glucose monitoring system.

To complete the chemo-optical glucose sensor, the glucose sensing enzyme GOx must be coupled with a dye which phosphoresces. This provides a means of optically measuring enzymatic activity, which could then serve as an indicator of real time glucose concentrations in an implantable glucose monitoring system. A Pd(II) pentafluorophenyl porphyrin dye has been chosen for the development of the chemo-optical sensor, and recent work shows promise for the incorporation of the oxygen sensing dye within the hydrogel based sensing system via encapsulation within micelles. Upon the creation of a hydrogel based system containing both the GOx functionalized PEG-NB microparticles and the micelle encapsulated Pd(II) porphyrin dye, a shell will be generated around the final structure in order to increase control of the rate of delivery of oxygen and glucose to the sensor. The platform offered by click hydrogels not only demonstrates the versatility of utilizing sequential click reactions for high stoichiometric control and binding specificity in forming a polymer matrix, but it also shows promise for the development of the next generation of glucose biosensors.

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