SEMI-PERMEABLE MICROCAPSULES FOR USE IN FLUORESCENCE-BASED GLUCOSE SENSING

An Honors Fellows Thesis

by

ELIZABETH GRACE JOACHIM

Submitted to the Honors Programs Office
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

April 2010

Major: University Studies Honors

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Approved by:

Research Advisor:	Michael J. McShane
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ABSTRACT

Semi-Permeable Microcapsules for Use in Fluorescence-Based Glucose Sensing.
(April 2010)

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Due to the pain associated with conventional blood sugar monitoring techniques and the rising prevalence of diabetes, the development of noninvasive glucose sensing techniques is desirable. Towards this aim, implantable fluorescence-based glucose sensors are being developed. One strategy used in these sensors is to create a competitive binding scheme between fluorescent-labeled dextran and glucose using a fluorescent-labeled binding protein, such as glucose binding protein (GBP) or Concanavalin A (ConA), so that the protein and dextran create a Fluorescence Resonance Energy Transfer (FRET) pair. The sensing chemistry is then encapsulated in microcapsules with walls of nanoscale thickness formed using the Layer-by-Layer (LbL) method. This work involved two principal objectives: 1) to measure the permeability of capsules comprising different materials to dextran, as a means of identifying materials that allow the diffusion of glucose into the sensor while preventing release of the other components; and 2) evaluating the effects of different core formation methods on encapsulation. Results indicate that adding salt to the LbL solutions can decrease the

permeability of the films to dextran and $[PAH\text{-}GPTS/PSS]_{10}$ films made with salt had the lowest overall diffusion coefficient. Also, both the time frame between core precipitation and the beginning of the LbL and the core precursor solution compositions affect encapsulation of ConA but not dextran. The development of the capsules described in this work represents an important first step towards the fabrication of a noninvasive glucose monitoring system.

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I would like to thank my lab mates, Jaebum Park and Dustin Ritter, for their immense and invaluable help with the diffusion experiments and capsule formation experiments respectively. I would also like to thank my biochemist friend, Jonathan Goodson, for answering all my ridiculous biochemistry related questions with such patience. Finally, I would like to thank my roommate, Kat Drinkwater, for driving me to and from campus late at night and tolerating my incessant research related complaints. This thesis would never have been finished without all of you.

NOMENCLATURE

C	Permeate concentration (normalized to feed concentration)
ConA	Concanavalin A
D	Diffusivity (cm^2/s)
d	Length of diffusion cell (cm)
FITC	Fluorescein isothiocyanate
FRET	Fluorescence Resonance Energy Transfer
GBP	Glucose Binding Protein
l	Thickness of nanofilm (cm)
LbL	Layer-by-Layer
t	Time (sec)
TRITC	Tetramethylrhodamine isothiocyanate

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CHAPTER I

INTRODUCTION

According to the American Diabetes Association, 23.6 million children and adults in the US have diabetes and it is the seventh leading cause of death [1]. An estimated $116 billion is spent annually to care for diabetics [1]. One of the keys to managing this disease is the accurate and regular monitoring of blood glucose levels. A number of products are commercially available for this purpose. These devices are typically invasive and require individuals to draw small amounts of blood for use in the test. Obviously, a less invasive, and less painful, method of blood sugar monitoring is desirable. Currently, there are very few minimally invasive blood sugar monitors on the market and all of them require daily calibration with traditional finger prick methods [2]. These systems are in need of improvement or replacement and, thus, new glucose monitoring methods are being investigated.

Recently, research has been moving towards fully implantable glucose sensors that can either be read through the skin using light or transmit their data to a device, perhaps using RFID technology [3]. The majority of glucose sensors currently being developed fall into two broad categories: electrochemical [4-6] and optical [7-10]. Within both of these categories there is an emphasis on the use of biological molecules as receptors as well as producing sensor components on the micro and nano scales. For optical biosensors, this trend generally involves housing the sensor elements, such as glucose

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This thesis follows the style and format of *IEEE Sensors Journal*.

oxidase and fluorescent dyes, within microcapsules or microparticles and using the Layer-by-Layer technique [11-15] to adjust sensor properties such as range and sensitivity. In electrochemical sensors, enzymes have been used as the sensing portion of carbon nanotube electrodes [16] and carbon nanotubes have also been used in non-enzymatic glucose electrodes [17].

For optical sensors, fluorescence schemes are the most prevalent. These sensors are typically based on oxygen-quenched dyes [18], environmentally-sensitive dyes [19], or FRET systems [20, 21]. Due to problems with tissue oxygen depletion *in vivo*, reaction byproduct formation, and enzyme degradation, competitive and non-competitive binding systems using a FRET pair or environmentally-sensitive dyes are desirable over enzymatic approaches.

Previous systems using competitive binding and FRET have been based on apoenzymes [20] or the binding protein Concanavalin A [21]. ConA-dextran systems often suffer from multivalent binding which lowers the percent change in fluorescence intensity when adding glucose [22]. This problem can be overcome, to an extent, by using apoenzymes, such as apo-glucose oxidase [23], or by using a dendrimer instead of dextran [22]. It has also been noted that ConA systems suffer from aggregation, a lack of specificity for glucose, toxicity, and degradation of reversibility over time [24, 25]. Apoenzyme systems do not suffer from the same shortcomings as ConA, being specific, reversible, and non-aggregating. Unfortunately, the process of removing an enzyme cofactor to make apoenzymes is not only time consuming and difficult, but the process

also destabilizes the enzyme making it less resistant to denaturation as well as introduces potentially toxic reagents, such as sulfuric acid, into the sensor [26].

In the past, a number of different non-competitive binding schemes have been proposed [12, 27, 28]. One such system used genetically modified GBP from *E. coli* and an environmentally-sensitive dye [19, 24]. Environmentally-sensitive dyes are generally considered superior to FRET pairs for use with GBP in non-competitive binding schemes [29]. FRET systems do not perform well because there is not enough of a conformation change in GBP upon binding of glucose to change the fluorescence intensity more than about 20% [29].

However, to our knowledge, *the effectiveness of FRET and GBP in a competitive binding scheme has not been evaluated.* Therefore, a competitive binding, FRET pair-based sensor utilizing a GBP mutant labeled with a fluorescent dye and fluorescent-labeled dextran encapsulated in microcapsules composed of polyelectrolyte multilayers is proposed here. As a first step towards the development of this novel system, the microcapsules previously described for macromolecule encapsulation [15] need to be studied further. And so, in this work capsule wall materials and core formation methods were investigated.

CHAPTER II

EXPERIMENTAL DESIGN

Materials

All chemicals were reagent grade, obtained from Sigma, and used as received with no further purification unless otherwise noted. Fluorescein isothiocyanate conjugated dextran of various molecular weights (FITC-Dextran), succinylated concanavalin A conjugated with tetramethylrhodamine isothiocyanate (TRITC-ConA, EY Laboratories Inc), D-(+)-glucose, hydrochloric acid 37%, calcium chloride dihydrate, sodium carbonate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), sodium chloride, (3-glycidyloxypropyl)trimethoxysilane (GPTS), sodium hydroxide, sodium phosphate dibasic, sodium phosphate monobasic, poly(allylamine hydrochloride) (M_w 15 kDa, PAH), poly(sodium 4-styrenesulfonate) (M_w 1 MDa, PSS), poly(ethyleneimine) 50% w/v in water (PEI). Other supplies included PES membrane filters (25 mm diameter, 0.02 μm pore size, Tisch Scientific) and open-faced filter holders (Pall Co).

The diffusion test bed, shown in Fig. 1, consisted of three 7 mL diffusion cells (PermeGear), stir plate, and a water circulator (Lab Companion). Fluorescence intensity measurements were made using a PC1 Spectrofluorometer from ISS (Urbana, IL) and an Infinite F200 plate reader (Tecan). Imaging was done with an Eclipse TE2000-U (Nikon) inverted microscope and Coolsnap HQ2 camera (Photometrics). Confocal imaging was done using a TCS-SP5 Spectral Laser Scanning Confocal Microscope

Fig. 1. Diffusion apparatus. This test bed includes three diffusion cells, a 96-well plate reader, and an automatic sampler.

equipped with an inverted DMI 6000 microscope and motorized stage (Leica). Particle sizing and counting was done with an Elzone II particle size analyzer (Micrometrics) fitted with a 190 µm orifice. A high frequency generator (Model BD-20, Electro-Technic Products) with a 115 V power supply (Electro-Technic Products) was also used.

Methods

Capsule formation and characterization

Capsule cores were formed by mixing a 200 mM calcium chloride solution with a solution containing 20 µM of 2 MDa FITC-Dextran and 200 mM sodium carbonate in 4 mg/mL PSS in a 1:1 ratio while stirring. The large molecular weight was chosen to slow diffusion. Cores were then transferred into a 50 mL centrifuge tube and centrifuged at 5000 RCF for 2 minutes. The supernatant was discarded before beginning LbL film assembly. The cores were alternately soaked in about 5 mL of a 2mg/mL polyanion and polycation solution for 10 minutes with an initial shaking of the tube to resuspend the particles. In between each polyelectrolyte layer, the samples were centrifuged at 5000 RCF for 2 minutes, the supernatant was discarded, and the samples were resuspended in

DI water for 5 minutes and centrifuged again. After 5 to 10 bilayers were deposited (always ending with PSS, the polyanion, as the outermost layer), the coated cores were resuspended in 10 mL of PBS (pH 7.2) for at least 24 hours. Finally, the cores were dissolved by treatment with 10 mL of alternating solutions of 0.1 M EDTA at pH 7.4 and 0.1 M HCl for 10 minutes each with rinsing and centrifuging at 2500 RCF for 10-15 minutes in between treatments. The reduction in centrifuge speed is to prevent capsule rupture. Both capsule cores and fully formed microcapsules were sized. Fluorescence and bright field confocal images of the microcapsules were taken after core dissolution.

Diffusion studies

Two variables, salt concentration and GPTS content, were investigated for their affects on dextran diffusion. It was hypothesized that adding salt to the polyion solutions would slow diffusion by making the films thicker and that adding GPTS to the films would also reduce leaching by making silane cross-linkages [30]. A total of four different compositions of LbL films were tested for their ability to inhibit FITC-Dextran diffusion: PAH/PSS with salt, PAH/PSS without salt, PAH-GPTS/PSS with salt, and PAH-GPTS/PSS without salt. The PAH solutions were at pH 2.3 and the PSS solutions were at pH 2.1. The salt concentration was 0.5 M NaCl and the GPTS concentration was 20 μL/mL.

The films were deposited on PES membrane filters following a 45 sec corona treatment and an initial PEI layer (2 mg/mL), which served as the first polycation layer. After corona treatment, substrates were placed in the open-faced filter holders so that the films

could be formed on only one side of the filter. About 1 mL of the polyelectrolyte solution was deposited on the substrate and allowed to soak for 7 minutes. Following soaking, the films were rinsed with DI water for about 45 seconds before applying the next polyion solution. Each film was composed of 20 total layers with the last layer being PSS. Completed films were stored in PBS buffer in parafilm sealed petri dishes. A total of 4 sets of films were prepared: three with salt and one without salt.

The diffusion properties of these four film configurations were tested in diffusion cells. PBS buffer was on the permeate side and a 0.2 μM solution of a medium weight FITC-Dextran (10.5 kDa) in PBS was on the feed side. Filters were oriented so that the film side of the substrate was oriented towards the permeate side to simulate diffusion out of a capsule (Fig. 2). The diffusion cells were continuously stirred and protected by a foil covering to prevent photobleaching throughout the duration of the experiment. Over approximately 55 hours, 200 μL samples were periodically taken with a pipette from both the feed and permeate sides. The samples were deposited in a 96 well plate, covered with foil, and stored at 4°C until the fluorescence intensity could be read at the end of the experiment. All the films were tested in diffusion cells about 24 hours after formation. One set each of salt and no salt films were also retested after soaking in PBS for 2 weeks in order to evaluate their stability over time.

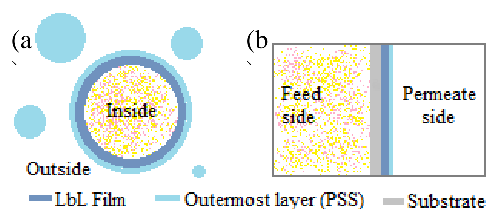

Fig. 2. Film orientation diagram. (a) microcapsules in solution (b) diffusion cell. The filters were oriented so that diffusion out of a capsule was modeled. This made the feed side of the cells representative of the

The diffusion coefficient, D, of each film tested was calculated using the linear portion of the generated plots according to the following equation:

$$D = l \times d \times \frac{dC}{dt} \qquad (1)$$

where d is the cell length in cm, l is the film thickness in cm, t is the time in seconds, and C is the normalized concentration of dextran.

Encapsulation methods

The standard core precursor solutions are 200 mM calcium chloride and 200 mM sodium carbonate with 4 mg/mL PSS. The materials to be encapsulated (FITC-dextran and TRITC-ConA) are then added to these solutions. Because binding proteins, like ConA, can form aggregates with dextran in solution, methods to prevent aggregation before core precipitation were tested. It was hypothesized that adding glucose to the precursor solution or putting the dextran and ConA in different precursor solutions would prevent this problem. A total of eight different encapsulation precursor solution configurations were tested (Table I). It was also hypothesized that immediately beginning LbL after core formation will prevent dextran and protein from leaching out

TABLE I
PRECURSOR SOLUTION CONFIGURATIONS

Precursor Solutions	Group A				Group B[a]			
$CaCl_2$	ConA, glucose, Dextran	ConA, Glucose	ConA, Dextran	ConA		Dextran		Dextran
Na_2CO_3, PSS		Dextran		Dextran	ConA, glucose, Dextran	ConA, Glucose	ConA, Dextran	ConA

[a]Group B configurations were made twice: once for immediate LbL and once for delayed LbL.

of the $CaCO_3$ matrix. To test this idea, four of the precursor configurations were tested at two different LbL start times: immediately and about 24 hours later (Table I).

For these studies, 1 mL of 0.2 μM 10.5 kDa FITC-Dextran in PBS, 2 mL of 1 μM TRITC-ConA in Tris buffer, and/or 50mM glucose were added to 10 mL of the appropriate precursor solution. The two solutions were poured into 50 mL centrifuge tubes and shaken for about 30 seconds to aid core formation. The cores were then centrifuged at 5000 RCF for 5-10 min. Some of the cores were immediately transferred to 2 mL microcentrifuge tubes for the LbL process while others were allowed to sit overnight without removing the supernatant. All cores were coated using a $[PAH\text{-}GPTS/PSS]_7$ scheme with 0.5 M NaCl in the polyelectrolyte solutions. For each layer 1 mL of polyion was allowed to adsorb for 7 min before being centrifuged for 2.5 min at 2500 g. About 1 mL of water was then added and the samples were vortexed for about 30 sec to resuspend the cores before centrifuging again. After 3-5 bilayers had been deposited, samples were sonicated for about 5 min to disperse aggregates that were forming. The coated cores were allowed to soak in PBS buffer for at least 12 hours so that the silane cross-linkages could form. The cores were dissolved using two treatments of 25 mL each of 0.1 M EDTA at pH 7.7. During each treatment the capsules were vortexed for 5 min, allowed to soak for 5 min, and vortexed another 5 min before being centrifuged at 5000 RCF for 10 min. After core dissolution, the microcapsules were transferred to cuvettes containing about 2 mL of PBS for fluorescent intensity readings. These readings indicate the relative concentrations of dextran and ConA that were encapsulated.

CHAPTER III

RESULTS AND DISCUSSION

Capsule formation and characterization

Particle size data indicates that the microcapsules are polydispersed, the majority of them being between 6 and 15 μm (Fig. 3). The distribution for uncoated cores (not shown) was similar, indicating that the films were very thin. Because of the thinness of the films, increasing the number of bilayers from 5 to 10 also does not change the particle size distribution. Fluorescent confocal images indicate that the microcapsules are often nonspherical and incapable of encapsulating dextran (Fig. 3 inset). Many of the capsules aggregate during the LbL process resulting in large, poorly formed microcapsules. If composed of only 5 bilayers, many of the capsule walls will not be contiguous thereby allowing some or all of the encapsulated material to escape. However, if the number of bilayers is increased to 7 to 10, a higher proportion of the walls will be contiguous.

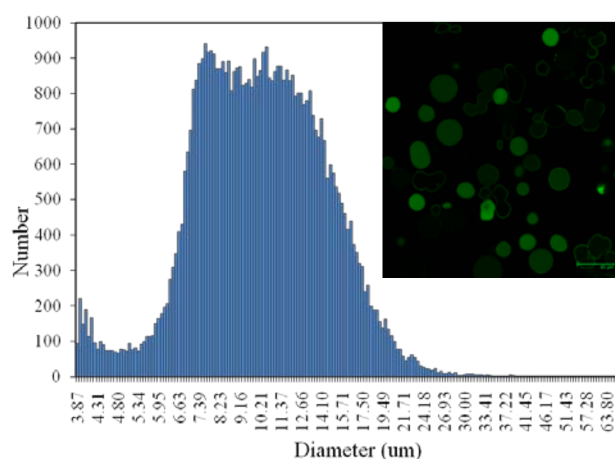

Fig. 3. Microcapsule size distribution: shows a lack of monodispersity in capsule diameters. Inset: confocal micrograph of microcapsules with $[PAH/PSS]_5$ walls showing some fully formed capsules, some shells with no dextran, and some misshapen capsules. Scale bar is 40 μm.

TABLE II
DIFFUSION COEFFICIENTS

Salt		No Salt	
PSS/PAH	7.4×10^{-13}	PSS/PAH	
	1.5×10^{-11}		1.9×10^{-10}
	4.2×10^{-12}		1.6×10^{-10}
	7.4×10^{-13}		
PSS/PAH-GPTS	5.3×10^{-13}	PSS/PAH-GPTS	
	7.1×10^{-12}		1.9×10^{-10}
	3.2×10^{-13}		1.2×10^{-10}
	2.2×10^{-13}		

All diffusion coefficients are in units of cm^2/s.

Diffusion studies

Diffusion results indicated that none of the films tested reached steady state after two days (Fig. 4). Furthermore, the salt films were still in the linear phase at this time. These data also show a change in the diffusion properties of the PAH/PSS films made with salt between the one-day and two-week testing times. The GPTS is most likely adding stability to the film preventing a change in diffusivity over time. According to the calculated diffusion coefficients, adding salt to the polyion solutions can decrease D by

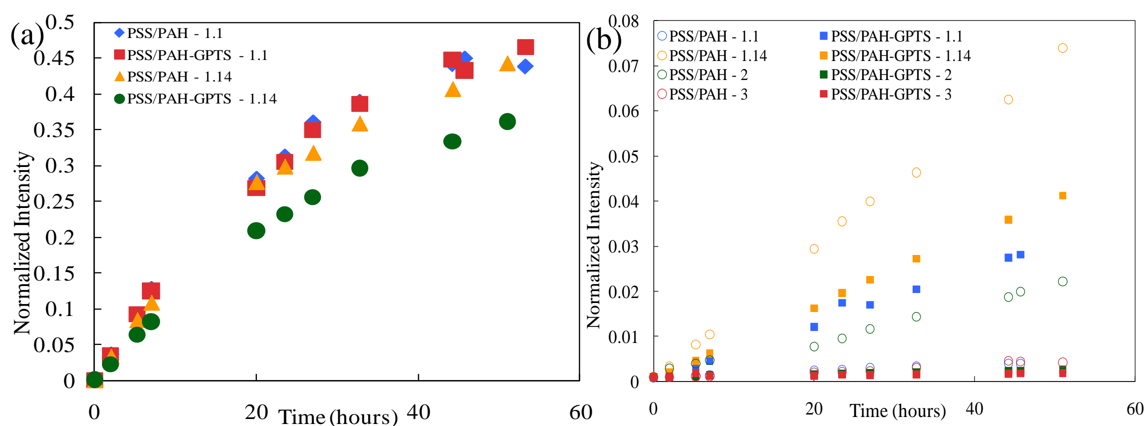

Fig. 4. Diffusion of dextran through films of various compositions. (a) films without salt in the polyion solutions (b) films with salt in the polyion solutions. Films 1.1 and 1.14 are the same film tested after 1 day in storage and 2 weeks in storage, respectively. All these intensity readings are from the permeate side and were normalized to the initial intensity in the feed side for a maximum value of 0.5.

at least one order of magnitude (Table II). Because the thickness of all the films was estimated to be about 100 nm based on previous ellipsometry measurements of the thickness for a $[PSS/PAH]_7$ film with salt [31], the calculated diffusion coefficients for the films without salt are under estimates.

Encapsulation methods

According to the ANOVA tests ($\alpha = 0.05$) conducted on the data from Fig. 5, both the precursor solution configuration and the time between core precipitation and commencement of LbL affect the final encapsulation of ConA ($p = 0.048$) but not dextran ($p = 0.0759$). Because the number of FITC and TRITC molecules is proportional to the number of dextran and ConA molecules respectively, the fluorescence intensity of the capsules is proportional to the amount of each molecule entrapped within.

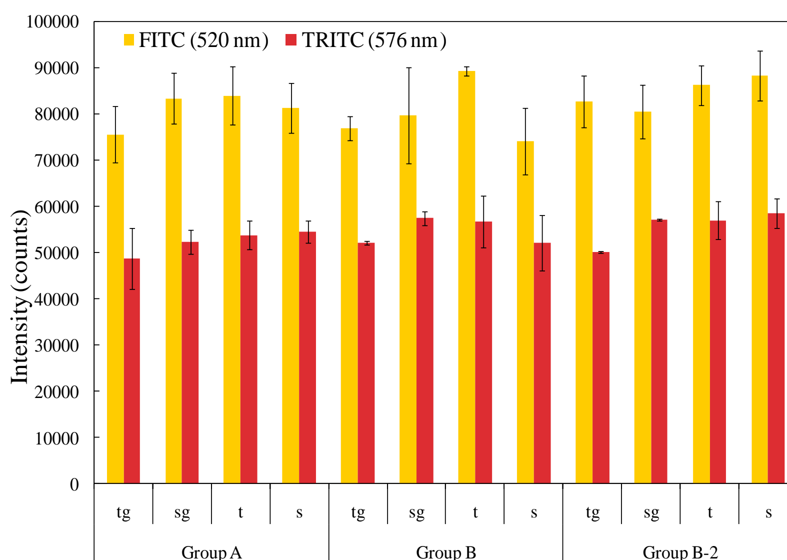

Fig. 5. Encapsulation of FITC-Dextran and TRITC ConA with varying precursor configurations. The groups are the same as in Table I with B-2 having a delayed LbL start. Dextran and ConA in the same precursor solution is denoted by t, in different solutions is s, and glucose in the ConA containing solution is denoted by g. Error bars represent 3 measurements of the same sample.

The Group A (ConA in $CaCl_2$) and Group B (ConA in Na_2CO_3) configurations showed slightly different patterns of encapsulation, especially in the glucose-free samples. Interestingly, these differences are not maintained when LbL is delayed (Group B2). In every group, the encapsulation is lowest when ConA, glucose, and dextran are mixed in the same precursor solution and relatively equal in all the other configurations.

CHAPTER IV

SUMMARY AND FUTURE WORK

The general structure and diffusion barrier properties of microcapsules constructed via LbL deposition of polyions around calcium carbonate cores were investigated. The capsules formed were neither monodispersed nor spherical. In addition, those made with only 5 bilayers were not able to fully entrap sensor components resulting in numerous empty capsules. By making capsule walls out of 7 to 10 bilayers of PAH-GPTS/PSS with 0.5 M NaCl the diffusion of dextran, a sensor component, can be limited.

Various core formation methods were also investigated. Based on preliminary results, it seems that both the precursor solution configuration and the time between precipitation and start of LbL can have an effect on final encapsulation of ConA but not dextran. The affects of the ConA containing solution ($CaCl_2$ vs. Na_2CO_3) are lessened when LbL is delayed. The delayed start time may allow for more $CaCO_3$ precipitation and thus greater total encapsulation. The least efficient encapsulation method is putting all three elements (ConA, glucose, and dextran) in the same precursor solution.

In the future, more LbL materials could be evaluated as dextran diffusion barriers. PSS and PAH were originally chosen for this study because they were the original materials used with this encapsulation technique [14, 15]. However, a similar technique for protein encapsulation used poly-L-lysine and heparin [12]. These so-called natural materials can

have the advantage of greater biocompatibility. Similarly, the reasons behind the ability of GPTS to decrease *D* and seemingly increase film stability needs to be studied. Also, higher molecular weights of dextran should be tested to determine the approximate molecular weight cutoff the films.

Now that general microcapsule structure and potential capsule wall materials have been identified, work on the other sensor components can begin. First, the *E. coli* glucose binding protein needs to be genetically modified to make it more amenable to fluorescent labeling by adding a sulfhydryl group. This group can be added by mutating an amino acid residue to a cysteine, which contains the group on its side chain. The free sulfhydryl group can then be reacted with an appropriate dye. Because GBP contains no native cysteine residues, the exact location of the dye binding site can be specified. One favorable site for mutation, residue 26, would locate the dye on the opposite face of the protein from the active site thereby preventing interference of the dye with the normal functioning of GBP. After producing sufficient quantities of Q26C GBP mutants, the binding affinity of GBP for dextran and glucose needs to be determined. Finally, sensors containing GBP and dextran will need to be fabricated and have their properties optimized using a combination of computer models and an *in vitro* test bed.

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