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CELL AND PROTEIN-BASED SENSING SYSTEMS FOR THE DETECTION OF ENVIRONMENTALLY AND PHYSIOLOGICALLY RELEVANT MOLECULES

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By
Kendrick Bruce Turner

Lexington, Kentucky

Director: Dr. Sylvia Daunert, Professor of Chemistry

Lexington, KY

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ABSTRACT OF DISSERTATION

Kendrick Bruce Turner

The Graduate School
University of Kentucky

2011

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The detection of small molecules in complex sample matrices such as environmental (surface and ground water, sediment, etc.) and biological (blood, serum, plasma, etc.) samples is of paramount importance for monitoring the distribution of environmental pollutants and their patterns of exposure within the population as well as diagnosing and managing diseases. Biosensors have demonstrated a singular ability to sensitively and selectively detect analytes in complex samples without the need for extensive sample preparation and pre-treatment. Nature has demonstrated myriad examples of exquisite selectivity in spite of complexity and we seek to take advantage of that attribute in the development of novel biosensing systems.

In the work presented here, we have developed both cell- and protein-based biosensing systems for the detection of hydroxylated polychlorinated biphenyls (OH-PCBs) and protein-based sensing systems for the detection of glucose. In the development of a whole-cell sensing system, the regulatory protein, HbpR, and its associated promoter was used to modulate the expression of luciferase. Additionally, the effector binding domain of HbpR, HbpR-A, was isolated and modified with a solvatochromic fluorophore resulting in a protein-based sensing system. For the detection of glucose, two different glucose binding proteins were engineered in an effort to tailor their characteristics, such as binding affinity and thermal stability, to develop a rugged, sensitive protein-based sensing system. We envision that these biosensing systems will find applications in the areas of environmental pollutant monitoring and the management and treatment of diseases such as diabetes.

KEYWORDS: biosensors, binding proteins, regulatory proteins, protein-based biosensors, cell-based biosensors

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CHAPTER ONE

INTRODUCTION

Portions reprinted from Turner, et al. *Cell-Based Bioluminescent Biosensors* in "Analytical Chemiluminescence and Bioluminescence: Past, Present and Future", A. Roda, Ed., Royal Society of Chemistry Press, Cambridge, United Kingdom, 2010

A biosensor is defined as a device that employs biological components such as proteins, tissues, organelles, nucleic acids, or whole cells to detect a physicochemical change and produce a measurable signal. Biosensors are typically composed of three parts: the biological sensing element, the signal transducing element, electronic and signal processing components and a display unit. The transducing element of a biosensor can produce a variety of signal outputs such as optical, piezoelectric, or electrochemical. Biosensors can be categorized as either molecular-based (binding proteins, enzymes, antibodies, aptamers etc.) or cell-based (whole cells, tissues, organisms, etc.). Biosensors can be designed with certain characteristics, which make them advantageous over traditional physicochemical analysis methods. These characteristics include high specificity/selectivity, ease of use, and the ability to provide relevant data related to the bioavailability of the target analyte in a given sample. Molecular-based biosensors offer the advantage of having generally faster response times than cell-based ones, although they are typically less rugged due to the often fragile nature of many isolated biomolecules and they fail to provide information on the bioavailability of the compound of interest.[1] Additionally, the production and isolation of biomolecules can be expensive and time-consuming. In contrast,

cell-based biosensors are usually more tolerant of extreme conditions, although, in many cases, they can require longer analysis times. Cell-based sensing systems also provide useful information on the bioavailability of the interrogated analyte and its ability to activate biochemical machinery, which can contribute to an increased understanding of the toxicity or physiological role of the compound of interest when this is, for example, an environmental pollutant or a biologically relevant molecule. As a result of their respective desirable properties, both cell- and protein- based biosensing systems are finding increasing application in the fields of environmental and clinical analysis, drug discovery, and toxicology, and are becoming the focus of much research to improve their characteristics and engineer them to respond to a greater variety of stimuli and analytes present in the environment.

DESIGN AND CONSTRUCTION OF BACTERIAL CELL-BASED BIOLUMINESCENT BIOSENSORS

Bacterial cell-based biosensors share a common basic design and include similar components. This basic design consists of an intact living cell containing a DNA sequence, in which the expression of a reporter gene/transducer is under the control of the promoter of a certain operon. Within this basic design, there can be much variation depending on the cells being used as a host, the type of promoter that is utilized (either constitutive or inducible reporter expression), and the detection strategy that is being employed.

As cells grow and are metabolically active, biosensing systems that employ a constitutive promoter to regulate the expression of the reporter protein, often present a basal expression of reporter, which produces a measurable signal even in the absence of the target analyte. This basal signal is reduced if the cells are subjected to stress as a result of exposure to toxic compounds or other adverse growing conditions. Thus, this kind of cell-based biosensors that employ constitutive promoters to regulate the production of a reporter protein can effectively provide information on the overall toxicity of a sample under investigation. However, it should be noted that such a system does not provide any information on the specific nature of the compounds that cause the toxicity to the cells or the sample.

In contrast, the gene fusion of an inducible promoter to a reporter gene yields a cell-based biosensing system in which the expression of a reporter protein occurs only in the presence of a desired condition (Figures 1.1, 1.2). Therefore, the presence of an analyte of interest activates the expression of a reporter protein resulting in an increase of the signal produced by the reporter. An inducible cell-based sensing system such as this is preferred when the goal is to detect and quantify a particular compound or condition in the sample. Furthermore, combinations of these genetic operons using different reporter genes under the control of different promoters can be achieved within a single organism and yield a single cell-based biosensor capable of responding to multiple analytes, an advantage when developing multiplexed assays.[2, 3] Inducible promoters are typically regulated by transcriptional regulatory proteins,

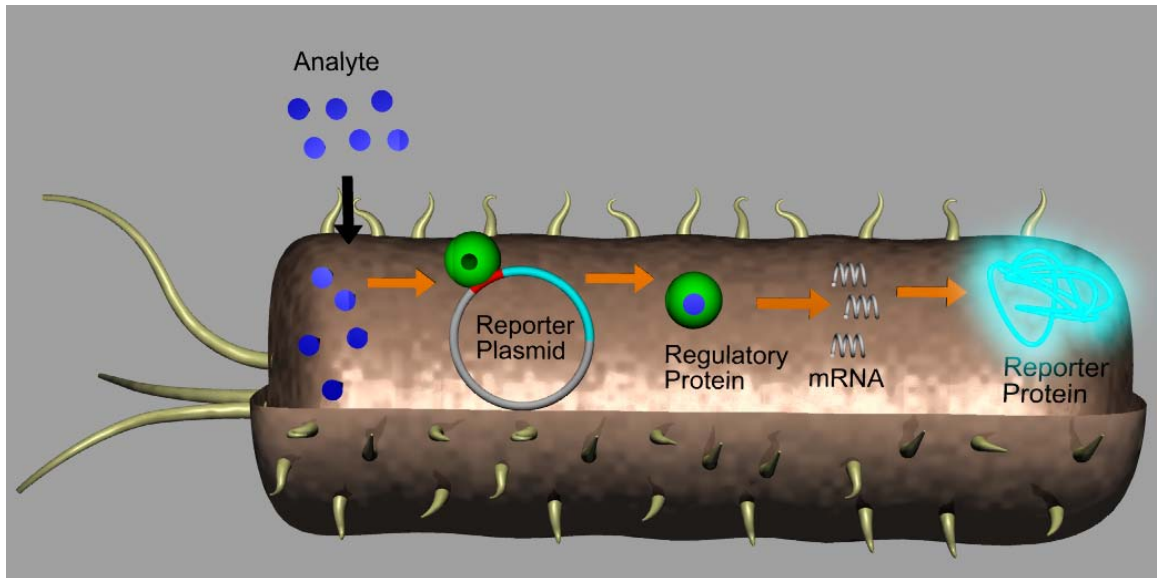


Figure 1.1 Schematic of an operon/regulatory protein cell-based biosensor featuring a negatively regulated operon. In the absence of the analyte, the regulatory protein is bound to the promoter on the reporter plasmid and prevents expression of the reporter protein. As the analyte concentration increases, it binds the regulatory protein, and the analyte-regulatory protein complex dissociates from the reporter plasmid. This triggers expression of the reporter protein, and a dose-dependent generation of the signal.

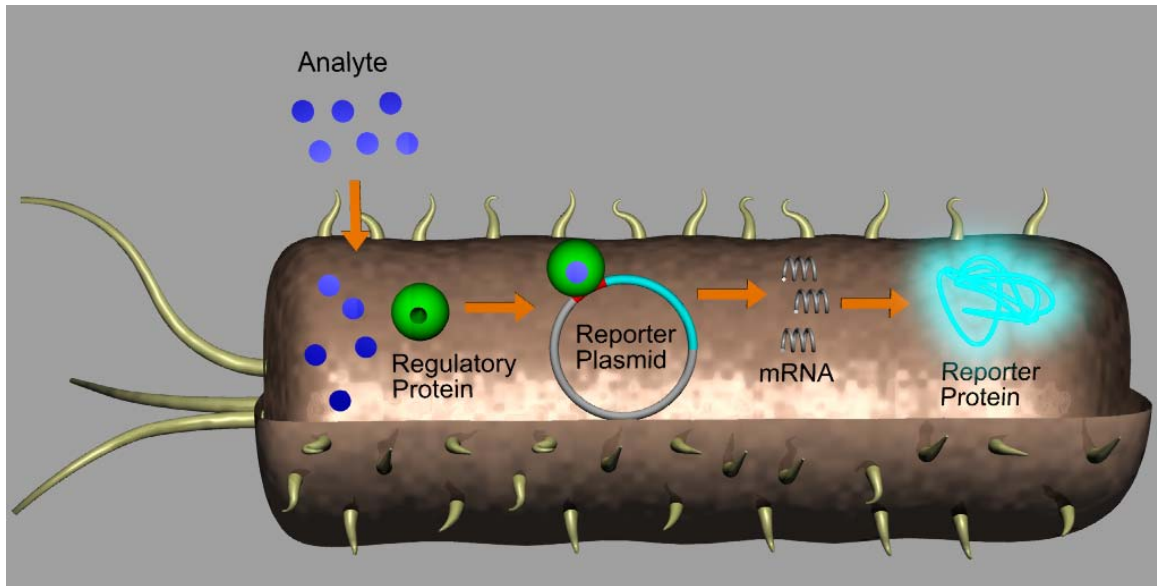


Figure 1.2 Schematic of an operon/regulatory protein cell-based biosensor featuring a positively regulated operon. When an analyte is present, it binds to the regulatory protein. This regulatory protein-analyte complex binds the promoter region on the reporter plasmid, thus triggering the expression of a reporter protein. This produces a luminescent signal which is dependent on the concentration of the analyte.

a kind of protein that evolved as a response mechanism of an organism to stress. In general, when an extracellular stimulus/compound acts upon the organism, these proteins are able to specifically or selectively recognize and bind to this compound, and subsequently respond by triggering the production of other proteins involved in defense mechanisms. These include the production of protein pumps that control the cellular efflux of the toxic compound(s), metabolic and synthetic pathway enzymes that degrade them, proteins that can sequester them, receptor proteins, etc.[1] These inducible-promoter regulatory circuits are advantageous to the host organism in that they ensure that transcription of their gene products occurs only when required to increase their survival. In the absence of an external stimulus, the regulatory circuit is turned off, no proteins are expressed from the genes, and the organism can conserve its resources and energy for other purposes.

In cell-based biosensing systems, recombinant DNA methods are employed to replace the gene products of the native operon with a reporter gene that produces a desired signal in response to the presence of a specific compound recognized by the regulatory protein. Thus, these systems combine the biospecific recognition afforded by the regulatory proteins with the signal generation stemming from the reporter gene. An example of the design of one such system is shown in Figure 1.3 featuring the *hbp* operon from *Pseudomonas azelaica*. The genes *hbpC*, *hbpA*, and *hbpD* of this operon are under the control of an operator/promoter that is regulated by the product of the gene, *hbpR*.

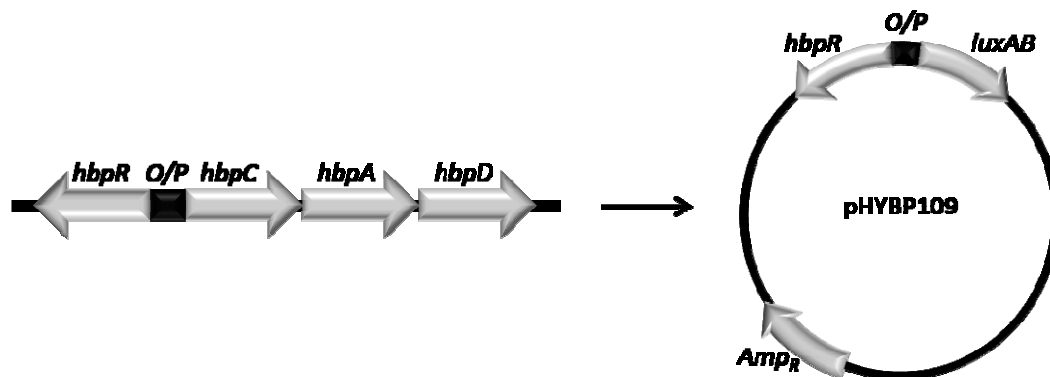


Figure 1.3 Design of the recombinant reporter plasmid component of a bioluminescent cell-based sensing system for hydroxylated polychlorinated biphenyls. The structural genes, *hbpC*, *hbpA*, and *hbpD*, of the *hbp* operon from the chromosome of *Pseudomonas azelaica* were replaced with the *luxAB* reporter gene in the construction of reporter plasmid pHYBP109. Upon transformation of pHYBP109 into *E. coli*, a cell-based biosensing system to detect various hydroxylated biphenyls in both buffer and serum was constructed.[4]

The enzymes, *HbpC*, *HbpA*, and *HbpD* are responsible for the degradation of 2-hydroxybiphenyl. A bacterial cell-based biosensor was constructed by replacing these structural genes with the reporter gene *luxAB*. Further study of this construct demonstrated that it responded to a variety of hydroxylated polychlorinated biphenyls (OH-PCBs) resulting in an increase in bioluminescence in a dose-dependent manner.[4]

REGULATION OF REPORTER GENE EXPRESSION

Within the realm of inducible cell-based biosensing systems, the reporter gene expression may be positively or negatively regulated. In a negatively regulated operon (Figure 1.1), a regulatory protein binds to an operator DNA sequence and prevents the expression of the reporter protein, with this binding being dependent on the presence or absence of the effector/analyte. In one instance, the regulatory protein is initially bound to the operator region, thus repressing the expression of the reporter. As the effector is added, it binds to the regulatory protein causing it to dissociate from the operator DNA sequence. This results in an increased expression of the reporter protein. The alternative is that the effector is required for binding of the regulatory protein to the operator. In this case, as the effector is added, a regulatory protein-effector complex forms, which binds to the operator resulting in a decrease in reporter protein expression.

Alternatively, in a positively regulated system (Figure 1.2) the operon regulation is mediated by a DNA sequence that acts as an “enhancer” of the system. There are two different mechanisms by which this regulation occurs: In

the first possible mechanism, the regulatory protein is bound to the enhancer, and consequently, produces a high level of initial expression of the reporter protein. As the concentration of the effector increases, it binds to the regulatory protein, causing its dissociation from the enhancer and, thus, reducing expression of the reporter gene. In the second case, the effector is required for the binding of the regulatory protein to the enhancer sequence. An increase in the effector concentration leads to the formation of a regulatory protein-effector complex which, in turn, binds to the enhancer DNA sequence and increases reporter protein expression.

From an analytical standpoint, it is desirable to have the lowest possible initial signal and an increasing dose-dependent response as the concentration of the analyte of interest increases. Thus, the most useful regulatory proteins are those from very tightly negatively regulated operons where the regulatory protein is initially bound to the operator and represses reporter protein synthesis. Accordingly, cell-based biosensing systems based upon these strategies, in general, afford the lowest limits of detection.

REPORTER GENES AND THEIR ATTRIBUTES

While the sensing element of a cell-based biosensing system determines the selectivity of its response, the reporter gene largely determines the sensitivity of the system. In that regard, the variety of reporter genes available is steadily increasing and each of these reporter genes possesses unique attributes that must be considered in the design of a biosensing system. The reporter gene of a

cell-based sensing system can yield a reporter protein that can generate a variety of different measurable signals. This chapter will focus on those producing an optical/luminescent signal. Optical signals are easily measured, and an extensive array of instrumentation is available, ranging from very sophisticated, state-of-the art to less complex, cost-effective hardware depending on the needs of a particular application. Additionally, a great variety of luminescent proteins have been well-characterized.[1, 5] A summary of the attributes of a variety of luminescent reporter proteins can be found in table 1.1. Following is a discussion of each of the types of luminescent reporter proteins and enzymes capable of generating an optical signal when employing an appropriate substrate.

A. Luciferases

The term luciferase encompasses a class of enzymes that catalyze a reaction in which a substrate known generically as a luciferin reacts with oxygen to produce light. These enzymes offer excellent limits of detection, largely in part because there is no background luminescence from endogenous activity in other organisms or from the media and samples in which the measurements are being taken. The high quantum efficiency of the bioluminescent reactions catalyzed by these enzymes also contributes to the low detection limits that they afford. Given that luciferase emits light via a biochemical reaction, there is no need for an excitation source, making the instrumentation needed for measuring bioluminescence simple and cost-effective. These are clear advantages of

Table 1.1 Common Reporter Proteins and their Characteristics.

Reporter Protein [reaction catalyzed] <i>Detection Method</i>	Advantages	Disadvantages
Bacterial Luciferase [FMNH ₂ + R-CHO + O ₂ → FMN + R-COOH + H ₂ O + <i>hν</i> (490 nm)] <i>Bioluminescence</i>	High sensitivity. May not require substrate. No endogenous activity in mammalian or bacterial cells. No light source needed.	Heat Labile. Narrow linear range. Requires aerobic environment.
Firefly Luciferase [Firefly luciferin + ATP + O ₂ + Mg ²⁺ → Oxyluciferin + AMP + P _i + <i>hν</i> (560 nm)] <i>Bioluminescence</i>	High sensitivity. Broad linear range. No endogenous activity in mammalian or bacterial cells. Spectral variants available. No light source needed.	Requires substrate. Requires aerobic environment and ATP. Requires solubilizers for substrate permeability into cells.
Renilla Luciferase [Renilla coelenterazine + O ₂ → Coelenteramide + CO ₂ + <i>hν</i> (480 nm)] <i>Bioluminescence</i>	No endogenous activity in bacterial or mammalian cells. No light source needed. Required Substrate is membrane permeable	Requires substrate and may require cell lysis.
Aequorin [Jellyfish coelenterazine + O ₂ + Ca ²⁺ → Coelenteramide + CO ₂ + <i>hν</i> (465 nm)] <i>Bioluminescence</i>	High sensitivity. No endogenous activity in mammalian or bacterial cells. No light source needed.	Requires substrate and the presence of Ca ²⁺ .
Green Fluorescent Protein [Formation of an internal chromophore Excitation/Emission: 395 nm/509 nm] <i>Fluorescence</i>	Autofluorescent. No substrate or cofactors needed. Spectral variants available. No endogenous activity in most systems. Stable at physiological pH.	Moderate sensitivity. Background fluorescence from some samples may interfere. Toxic to some cell types. Formation of chromophore can be slow.
β-Galactosidase [Hydrolysis of β-Galactosides] <i>Chemiluminescence</i> <i>Fluorescence</i> <i>Colorimetric</i> <i>Electrochemical</i>	Sensitive and stable. Moderate linear range. Can be used in anaerobic environment	Endogenous activity. Requires substrate.

luciferases over other luminescent systems such as those based on fluorescence and therefore, make this family of enzymes great candidates for use as reporters.

Luciferases have been isolated from a number of organisms, which include fireflies, bacteria, worms, fungi, etc. Commonly used as reporter genes in bacterial cell-based biosensing systems are those isolated from bacteria including members of the genera *Vibrio*, *Xenorhabdus*, and *Photobacterium*.^[1] In these organisms, bioluminescence is conferred by the *lux* operon which is comprised of *luxCDABE* gene cassette. Of the five genes in the *lux* operon, only *lux A* and *lux B* (with the addition of a suitable substrate) are required for bioluminescence. The products of these two genes form the catalytically active dimer, which oxidizes a long-chain aliphatic aldehyde (luciferin) and a reduced flavin mononucleotide (FMNH₂) cofactor to the corresponding carboxylic acid and FMN, respectively. During this process, an unstable complex containing an excited oxide bond is formed, which relaxes to the ground state and the concomitant emission of light at 490 nm.^[6]

While the *luxAB* genes alone, employed as a reporter, are enough to produce bioluminescence, the addition of a long-chain aldehyde substrate, usually decanal, is required unless the entire *luxCDABE* gene cassette is present. The enzyme products of the remaining genes in the cassette, *lux C*, *lux D*, and *lux E* provide the aldehyde substrate needed for bioluminescence. However, it has been noted that the use of *luxAB* alone results in increased bioluminescence and, consequently, improved sensitivity of measurement in a biosensing system.^[7] This is likely the result of limited substrate production from

the products of the *lux* cassette as opposed to the excess of substrate added when *luxAB* is used. The design of a cell-based biosensing system using the *luxCDABE* is advantageous in the development of simplified assays and applications to be used in real-time monitoring. For applications requiring increased sensitivity or improved control over when the generation of the bioluminescence signal occurs, the *luxAB* reporter gene is preferred.

In addition to bacterial luciferases, firefly luciferase has also been well-characterized and widely used as a reporter gene. Firefly luciferase is a 62 kDa monomer that catalyzes the oxidation of a benzothiazolyl-thiazole luciferin to oxyluciferin in the presence of ATP, oxygen, and Mg^{2+} . The oxyluciferin produced is an excited molecule that subsequently relaxes to a ground state resulting in the emission of light at a maximum wavelength of 550-575 nm. Firefly luciferase has approximately 10-fold higher quantum yield than bacterial luciferase, which endows it with a broad dynamic range (7-8 orders of magnitude).[8, 9] Additionally, mutagenesis of firefly luciferase has resulted in enzymes that emit light at wavelengths in a wider range of the visible spectrum allowing for the development of multiplexed analysis.[10] Examples of these include red-shifted, thermostable variants of luciferase from *Photinuspyralis*. [11] However, the need for the addition of a substrate and the requirement for ATP can, in some cases, limit the application of firefly luciferase.

Another luciferase well-studied as a bioreporter was isolated from the marine organism *Renilla reniformis*, a species of sea pansy. Renilla luciferase is a 31 kDa monomeric enzyme that catalyzes the oxidation of coelenterazine and

results in the emission of light at 480 nm.[12] The sensitivity and dynamic range of Renilla luciferase is similar to that of firefly luciferase; however, Renilla luciferase is not as widely used as a bioreporter.[5, 13] In applications where multiplexing or a dual-reporter based system is desirable this luciferase is sometimes used along-side firefly luciferase as they have distinct wavelengths of emission. However, the need for substrate addition and cell lysis make using either firefly or Renilla luciferases less appealing than bacterial luciferase since increased cost, time, and error is introduced as a result.

Luciferases from other organisms have been successfully used as bioreporters in cell based sensing systems. In work by Wu et al., a dual-reporter system was constructed by transforming NIH 3T3 fibroblast cells with a plasmid containing *Cypridina* luciferase (CLuc) fused to a target gene and a plasmid containing *Gaussia* luciferase (GLuc) as a control plasmid to monitor gene expression in these cells.[14] Both CLuc and GLuc are secreted into the growing medium upon expression and emit at different wavelengths (460 nm and 480 nm, respectively), allowing for simultaneous measurement without cell lysis.

The variety of luciferases is increasing as novel proteins are being identified and integrated as reporters into cell-based sensing systems. For example, the recently characterized luciferases from the organisms *Luciola italica*[15] and *Phrixothrix hirtus*[16] are expanding the palette of emission wavelengths available when selecting reporter proteins. Two other novel luciferases from the marine copepod, *Metridiapacifica*, are thermostable bioluminescent proteins with distinct emission kinetics and are efficiently

secreted into culture medium upon expression as a result of the presence of an N-terminal signal peptide.[17] Because these are secreted into the culture medium, they could be incorporated into a cell-based sensing system and continuously monitored in a portion of culture medium independent of cell lysis.

B. Aequorin

Aequorin is a 22 kDa photoprotein native of the marine jellyfish *Aequorea victoria*. The emission of bioluminescence by aequorin is different from that of the luciferases, including *Renilla* luciferase. Aequorin needs an organic imidopyrazine substrate, coelenterazine, and the presence of Ca^{2+} for emission of bioluminescence. Coelenterazine resides within a hydrophobic pocket within the structure of the protein, while Ca^{2+} binds to three conserved EF-hand regions of aequorin. It is the binding of aequorin to Ca^{2+} that causes the protein to undergo a conformational change, which causes coelenterazine, in the presence of molecular oxygen, to go through an excited state from which it relaxes to form coelenteramide and emit bioluminescence at 460-470 nm.[1] The emission from aequorin follows flash-type kinetics with an emission from the native protein lasting about 3 seconds and a quantum yield of 0.15.[18] Mutagenesis of aequorin has led to the development and characterization of mutants with tuned emission lifetimes and altered wavelengths allowing for multiplexing in both time and spatial (wavelength) domains.[19] While aequorin has found some application in cell-based biosensing systems, its use has been somewhat limited, due mostly to its sensitivity to the presence of calcium ions and the need for the

addition of a substrate. In spite of these limitations, aequorin has been employed in high-throughput screening assays where its sensitivity to calcium is imperative in the study of G-protein coupled receptors and in screening compounds that act as their agonists or antagonists.[20] Also, the detection of specific pathogens, such as *Yersinia pestis* and *Bacillus anthracis*, has been achieved using a sensing system named CANARY (Cellular Analysis and Notification of Antigen Risks and Yields).[21] This system is constructed by engineering B cells that express both aequorin as a bioreporter and membrane-bound antibodies for the pathogen of interest. Even when exposed to low levels of pathogen, the antibodies are capable of recognizing their target. The resulting binding event triggers an increase in intracellular calcium concentration, which leads to the subsequent bioluminescence emission by aequorin within seconds. Novel relatives of aequorin, such as the photoprotein, clytin, from *Clytia gregarium* have been recently characterized and found to be less sensitive to calcium ions.[22] The availability of spectral variants for multiplexing, exceptional sensitivity (in the sub-attomole range), and lack of endogenous expression in other organisms warrants consideration of aequorin as a reporter gene.

C. Green Fluorescent Protein (GFP)

Like aequorin, the green fluorescent protein originates from the jellyfish *Aequorea victoria*. However, unlike the bioluminescent reporter genes mentioned thus far GFP emits fluorescence. Native GFP is a 238 amino acid protein possessing a β -barrel structure containing an internal fluorescent

chromophore. This chromophore is formed from three amino acids (threonine 65, tyrosine 66, glycine 67) in the interior of the protein by cyclization and oxidation of the tripeptide upon proper protein folding. The exceptional stability of GFP allows for the accumulation of reporter protein, which is particularly relevant when used in bacterial cell-based biosensors employing a weak promoter. GFP does not require a substrate to fluoresce, however as with any fluorescent reporter, it requires irradiation at its excitation wavelength maximum of 395 nm, resulting in light emission at a wavelength of 509 nm with a quantum yield of 0.88.[6, 23] Extensive research has generated a large number of GFP mutants with altered excitation and emission maxima, stabilities, and signal intensities.[8, 24, 25] GFP is also tolerated by a variety of cell types, including mammalian and bacterial cells. However, because of interference from background fluorescence in samples, the detection limits afforded by GFP are not comparable with those of bioluminescent proteins such as the luciferases or aequorin.[1]

D. β -Galactosidase

The gene product of the *lacZ* gene from *Escherichia coli* is β -galactosidase, an enzyme whose biological function is to cleave lactose into galactose and glucose, although it has been shown that the enzyme may act on a variety of substrates. Depending on the substrate employed, β -galactosidase can generate a fluorescent, chemiluminescent, colorimetric, or electrochemical signal.[25] For the production of chemiluminescence signals, 1,2-dioxetane

derivatives are typically used as a substrate. As a reporter gene, *lacZ* can afford detection limits as low as 2 fg with a dynamic range of 5-6 orders of magnitude.[1, 5] Despite these advantages, the need for the addition of a substrate and the requirement for cell lysis to make the substrate available to the enzyme restrict the use of *lacZ* as a reporter gene for certain specialized applications.

ADVANTAGES AND LIMITATIONS OF CELL-BASED BIOLUMINESCENT BIOSENSORS

The continuous discovery of new reporter molecules and recognition elements by biologists along with advancement in the field of recombinant DNA broadens the range of types of sensors that can be developed with regard to selectivity, sensitivity, and parallel analysis. Moreover, the identification and investigation of organisms that can be employed and survive in extreme environments, such as extremophiles and spore-forming microorganisms, should result in systems that present expanded storage and working conditions and are more resilient to extreme settings. This improved ruggedness and storage of cell-based biosensors should make them more amenable to field-portable environmental and clinical applications.[26]

Uniquely among biosensing systems, cell-based systems can provide significant information regarding the bioavailability of the compound being interrogated.[27] When employing an intact cell in the sensing system, the

compound being detected must be transported into the cell and activate certain cellular processes and pathways in order to produce a response. In this regard, a cell-based sensing system can identify those species, to be transported across the cell membrane. This information is especially useful in determining relevant toxicological characteristics of complex mixtures in which some components may be bioavailable while others may not. The bioavailable concentration of a species as detected using a cell-based biosensor is often related to the total concentration determined by standard physicochemical analysis to better characterize the sample being analyzed.[28]

While these advantages warrant further study and development of cell-based biosensing systems, there are a number of limitations that must be overcome before their full potential as an analytical method can be realized. Inherent to all biological systems is some degree of variability; cell-based biosensing systems are not immune to this. This variability can result from growth of the cells in non-ideal conditions, response to various components in complex samples, or a number of other unidentified factors. This variation can contribute to inter- and intra-assay variability. To address this issue, cell-based systems have been developed that carry a secondary plasmid in which a unique reporter protein is under the control of a constitutive promoter.[29] This allows for the response from the analyte-inducible promoter to be normalized with respect to cell growth and metabolism.

As previously mentioned, cell-based biosensing systems provide bioavailability information. However, when bacterial whole-cell sensing systems

are employed, this does not lead to a direct correlation to all relevant toxicological information as it applies to higher organisms such as humans. This can be addressed by the development of biosensing systems based on more complex cell types such as yeast or mammalian cells, thus giving data that is more applicable to these more complex organisms. Also, further study of similarities in biochemical pathways between cell types used in biosensing systems and cells present in higher organisms may lead to more accurate extrapolation of relevant toxicological data.

Finally, there can be some degree of instability in the plasmid DNA within cell-based sensing systems resulting in decreased reproducibility of measurement. This can occur as the cells carrying the exogenous genetic material reproduce and replicate the plasmid DNA contained within them. While the rate of error is very low, some mutations can occur. To negate this effect, plasmid DNA can be integrated into the chromosome of the cells being used, resulting in increased genetic stability.

APPLICATIONS OF CELL-BASED BIOSENSING SYSTEMS

The distinct properties and advantages discussed previously have allowed the application of cell-based biosensing systems in different fields. The use of bioluminescent reporters allows for compact, portable instrumentation due to the lack of need for an excitation source, a requirement in fluorescence measurements. In addition, bioluminescent bioreporters lack the background signal deriving from fluorescence generated by other components in the sample

matrix when exciting fluorescent reporters, which contributes to their superior sensitivity. Many specific examples can be found throughout the current literature describing applications in the realm of on-site environmental monitoring, drug candidate screening, clinical testing, high-throughput screening, etc. Recent examples of these applications will be highlighted here in more detail. Additionally, a number of bacterial cell-based biosensing systems are commercially available; examples of these are shown in table 1.2.

Cell-based biosensors have been engineered that can determine factors such as general stress, oxidative stress, and genotoxicity. In these constructs, a reporter gene is placed under the control of a promoter capable of responding in a dose-dependent manner to one of these stressors. Such systems have been developed to monitor oxidative stress,[2, 30] protein damage,[30, 31] DNA damage,[2] among others.

Bacterial cell-based biosensors are commonly used in environmental monitoring. Typically, soil and water samples concerning environmental contamination are complex in nature, containing both naturally occurring and foreign components. The specificity of the biological recognition element in a cell-based biosensing system is ideally suited for detecting a desired compound in a complex mixture. To that end, biosensors have been developed for a variety of analytes ranging from metals to organic pollutants and representative examples of these are discussed below.

Table 1.2 Commercially available bacterial cell-based biosensing systems

Product Name [Responds To]	Description	Manufacturer	Reference
Microtox™ [General Toxicity]	Inhibition test based on freeze dried <i>V. fischeri</i>	Strategic Diagnostics, Inc.	http://www.sdix.com/
Mutatox™ [Mutagenic Toxicity]	Engineered dark variant of <i>V. fischeri</i> recovers luminescence restored upon exposure to mutagenic compounds	Strategic Diagnostics, Inc.	http://www.sdix.com/
BIOMET™ [Zn, Cd, Cu, Ni, Pb, Cr, or Hg]	Engineered, metal-tolerant <i>Ralstoniametallidurans</i> produces luciferase in response to metals	Vito	http://wwwa.vito.be/english/index.htm
Cellsense™ [Chlorophenols, other organics]	Engineered <i>E. coli</i> produces amperometric response to analytes		Farre, <i>et al.</i> [32]
BioTox™ [General Toxicity]	Inhibition test based on freeze dried <i>V. fischeria</i> tailored for sediment samples	Aboatox	http://www.aboatox.com/
LumiStox™ [General Toxicity]	Inhibition test based on frozen <i>V. fischeria</i>	Hach Lange	http://www.drlange.com

There are a number of reports in the current literature regarding cell-based assays for the detection of inorganic analytes, specifically metals. The design of these biosensors is based upon the use of regulatory elements from microorganisms, which natively regulate the expression of genes to confer metal resistance, to control the expression of a bioreporter instead. Sensing systems have been developed for a variety of environmentally relevant toxic metals and metalloids including mercury,[33-35] antimonite/arsenite/arsenate,[36-39] cadmium,[36, 40, 41] chromate,[42] and aluminum[43] among others. In addition, inorganic compounds, such as nitrate, have been detected using cell-based sensing systems.[44] Detection limits as low as femtomolar with analysis times as short as 30 min have been reported for these species. Progress towards the development of a portable biosensing system for the detection of metals has been achieved by the engineering of a fiber-optic device consisting of mercury and arsenic sensing bacterial biosensors immobilized on optical fibers.[45] Environmentally relevant detection limits were obtained using this biosensing system: 2.6 µg/L for mercury, 141 µg/L for arsenic(V), and 18 µg/L for arsenic(III).

Cell-based biosensing systems have been developed for a number of organic compounds including endocrine disrupting compounds (EDCs), polychlorinated biphenyls (PCBs) and their metabolites, phenol, catechols, naphthalene/salicylic acid, benzene/toluene/ethylbenzene/xylene, etc.[5, 46-52] Many of these compounds are nearly ubiquitous in the environment and pose negative health effects on many organisms, including humans. They share

structural similarities and biological activities with naturally occurring compounds such as hormones. Traditional quantification of these compounds from environmental samples involves extensive sample pretreatment, derivatization, and extraction prior to detection by a suitable instrumental method. In addition to environmental samples, it has been demonstrated that a number of varied analytes can be detected in biological samples such as blood serum, making whole-cell sensing systems useful in the detection of biomarkers of exposure as well.[4]

Rather recently, cell-based biosensors have been developed for quorum sensing signaling molecules, the integral elements of the bacterial communication system. Quorum sensing is a phenomenon in which certain bacteria communicate by producing, secreting, sensing and responding to signaling molecules. The concentration of these molecules correlates to the density of the cells. This cell-to-cell communication allows the organisms to control the expression of specialized proteins depending on the cell population size. Since bacterial processes, such as, production of virulence factors, formation of biofilms and ability to colonize a certain environment are regulated by quorum sensing, the detection and quantification of quorum sensing signaling molecules may be relevant in the investigation of the status of a variety of diseases that have been linked to bacteria.[53] Cell-based biosensors have been developed by placing the expression of a bioreporter under the control of promoters and associated recognition/regulatory proteins from bacterial quorum sensing regulatory systems that respond to the presence of quorum sensing

molecules.[54] Successful use of these sensors has also been demonstrated in biological samples such as saliva and stool.[55]

Cell-based biosensors also exist for a number of antibiotic compounds. As the use of antibiotics increases, antibiotic resistance mechanisms in microorganisms are becoming increasingly widespread. Antibiotics are also being found in environmental and food samples; for instance, they have been detected in chlorinated drinking water at trace levels (down to $\mu\text{g/L}$ levels).[56] Conventional methods to detect antibiotics rely on immunoassays, chromatographic methods, and microbial growth inhibition tests. Because of their unique properties, especially the ability to characterize the bioavailability of an analyte, cell-based biosensors are well-suited for these applications. Such biosensors have been developed for the detection of antibiotics in various types of samples such as water and food products,[57] as well as blood and serum.[58] Cell-based biosensors have been used for the determination of antibiotic activity on a number of biochemical pathways in tandem with the screening of natural products for antibiotic activity. For example, a panel of five gene promoter regions from the soil bacterium *Bacillus subtilis*, which have altered mRNA expression profiles upon antibiotic exposure, were fused to a firefly luciferase reporter gene.[59] These genes, *yorB*, *yvgS*, *yheI*, *ypuA*, and *fabHB*, participate in biosynthetic pathways such as the synthesis of DNA, RNA, proteins, cell wall, and fatty acids, respectively. Biosensors based on the use of these genes along with luciferase have been used in a high-throughput screening mode to investigate the antibiotic activity of 14,000 natural products. Bacterial cell-based

biosensors have also been developed to examine the microbicide activity of some antibacterial polymers. Luminescence produced by the *E. coli* strain O157:H7 modified to express bacterial luciferase via the *luxCDABE* gene was monitored upon exposure to polymer compounds to determine bactericidal properties.[60] Bacterial biosensors also exist for the screening of antimicrobial activity of compounds in the gas phase that may find use in sterilization procedures. To that end, the bioluminescent bacterium *Pseudomonas fluorescens 5RL* was immobilized on a 0.2 μm membrane filter and exposed to varying concentrations of chlorine dioxide gas. In this system, a decrease in luminescence was correlated to increased antimicrobial activity of the gas.[61]

The detection of other biologically relevant molecules, such as sugars has also been achieved using a cell-based biosensor approach. Cell-based biosensing systems have been developed for a number of sugars by placing the expression of a reporter gene under the control of a promoter and regulatory protein responding to the desired sugar. For example, cells have been engineered to detect arabinose,[3, 62] glucose,[63] sucrose,[63] and lactose[3, 63]. Multi-analyte detection has been demonstrated with these compounds with the simultaneous detection of lactose and arabinose using a single biosensing organism in which two variants of GFP with distinct emissions were used as reporters for each sugar.[3] Cell-based biosensors are especially useful in the detection of sugars as they proved certain advantages over conventional detection methods. For example, detection of sugars using electrochemical

methods often suffer from a lack of specificity and those based on spectroscopic methods require derivatization of sugar with a chromophore substrate.

Applications of bacterial cell-based biosensors can also be found in the field of molecular biology. The *luxCDABE* cassette from *Photobacterium luminescens* was cloned into a pCRII vector and transformed into *E. coli*. The resulting cells were then grown with varying concentrations of lytic bacteriophage T4 at varying temperatures. The bioluminescence was monitored with respect to time, and as the bacteriophage lysed the bacteria a decrease in bioluminescence was observed. The results allowed the quantitation of the bacteriophage as well as determination of its thermal deactivation conditions.[64]

In the field of medicine, genetically engineered bacterial cell-based biosensors have found applications in imaging *in vivo* and *in vitro*. *E. coli* engineered to express GFP in response to quorum sensing molecules, *N*-acylhomoserine lactones (AHLs), were introduced into mice that had been infected with *Pseudomonas aeruginosa*, an opportunistic pathogen that uses these molecules for intercellular communication.[65] The lung tissue from the mice was examined by confocal scanning laser microscopy and the pathological damage observed was correlated to fluorescence measured as a result of increased AHL levels from bacterial presence. *E. coli* that migrate preferentially to tumor tissue, harboring a plasmid containing the *luxCDABE* cassette, were injected into mice with CT26 mouse colon cancer and the subsequent bacterial migration was imaged by detection of bioluminescence from the luciferase expressing bacteria.[66] The ability to image tumors *in vivo* in a non-invasive

manner is a valuable tool when diagnosing and monitoring the spread of a different types of cancer, as well as when screening for novel antitumor drugs. Rather recently, Foucault *et al.*, engineered *E. coli* to express either bacterial luciferase or mutants of firefly luciferase that were employed for real-time *in vivo* monitoring of infection in mice.[67]The use of bioluminescent bacterial biosensors in these applications offers a number of advantages, perhaps the most important being the lower detection limits due to the lack of background emission associated with similar methods employing fluorescent reporters.

DESIGN STRATEGIES OF PROTEIN-BASED BIOSENSORS

Protein-based biosensors, in contrast to cell-based biosensors, consist of an isolated protein as the recognition element coupled directly to a signal transducing element. The protein employed can be a small-molecule binding/transport protein, an antibody, a regulatory protein, an enzyme, or a small peptide fragment derived from functional proteins. The signal output for these biosensing systems can be electrochemical[68, 69], fluorescent[70], phosphorescent[71], bioluminescent[72], or piezoelectric[73], etc. The work discussed here will focus on those protein-based biosensing systems with an optical (fluorescence/bioluminescence) signal output. Because isolated proteins are employed, the assay time is limited only by the timescale of the recognition/binding event, leading to shorter assay times when compared to cell-based biosensing systems. Additionally, much of the natural variability inherent

in using metabolically active whole-cells as a sensing element can be reduced or eliminated when using isolated proteins.

Protein-based sensing systems can be broadly placed in two categories: genetically-encoded biosensing systems and chemically-modified biosensing systems (Figure 1.4). In the genetically encoded approach, the protein is engineered in such a way that the isolated product contains both the recognition element and the transducing element without need for further modification. A typical example of a genetically encoded biosensing system is a fusion of a ligand binding protein to reporter protein, such as GFP, in which a change in fluorescence is observed upon ligand binding. Chemically-modified biosensing systems must be further modified after the protein purification step. Usually, a sensing protein is modified by covalent attachment of a small reporter molecule. An example of a chemically-modified protein-based sensor is a ligand binding protein in which an organic fluorophore is covalently attached to a site on the protein such that a change in fluorescence can be measured upon ligand binding. Following is a more in-depth discussion of each of these types of sensing systems.

A. Genetically-encoded Biosensing Systems

Genetically encoded protein-based biosensing systems generally consist of a single protein or a fusion of two or more proteins that, upon purification, contains both the sensing and transducing elements without the need for further modification. The most common application for these systems is for *in vivo*

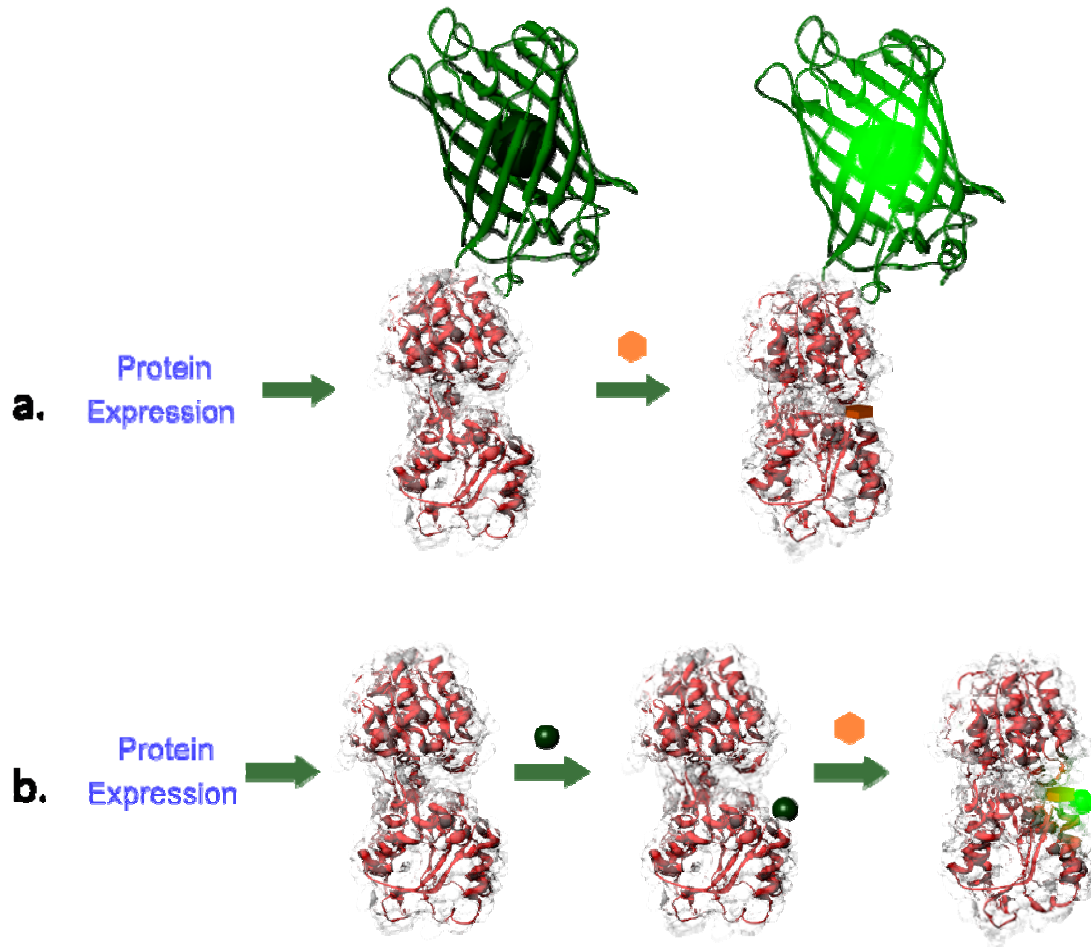


Figure 1.4 Comparison of genetically-encoded and chemically-modified protein-based biosensors. In genetically encoded biosensors (a.), a fusion of a sensing protein (red) to a reporter protein (green), results in a functioning biosensor for a desired analyte (orange) following protein expression. In contrast, chemically modified biosensors (b.) consist of a sensing protein which is covalently modified after expression with an appropriate reporter molecule (green), which yields a functioning biosensor.

imaging for the monitoring of various cellular processes, and there are myriad of examples in the literature for their uses.[74, 75] Genetically-encoded systems are uniquely suited for imaging applications because their spatiotemporal activity can be tailored based on the selection of proteins used as the molecular recognition element and reporter. However, these systems have also found some application in *in vitro* sensing applications due to relative ease of design and preparation when compared to chemically-modified biosensors.[72, 76]

There are a number of well-studied strategies used in the development of genetically-encoded biosensing systems (Figure 1.5). The most common reporter molecules employed in the design of genetically-encoded systems are GFP and its variants and aequorin, thus the examples discussed herein will focus on these proteins. In the first strategy, an intramolecular Förster Resonance Energy Transfer (FRET) approach is used. Briefly, FRET is a phenomenon in which a donor molecule is excited and then nonradiatively transfers a portion of its excited-state energy to a proximal (typically 10-100 nm) acceptor molecule which is in turn promoted to an excited state and undergoes fluorescence. Upon analyte binding, a conformational change occurring in the binding region results in a change in the distance between the donor and acceptor. This change in distance manifests as a change in FRET efficiency resulting a change in signal.[77] The second strategy is also FRET-based, but relies instead on an intermolecular FRET approach. In this strategy, the donor and acceptor are located on two different proteins. Upon the binding or association of these two proteins, the donor and acceptor are brought closer together, leading to an

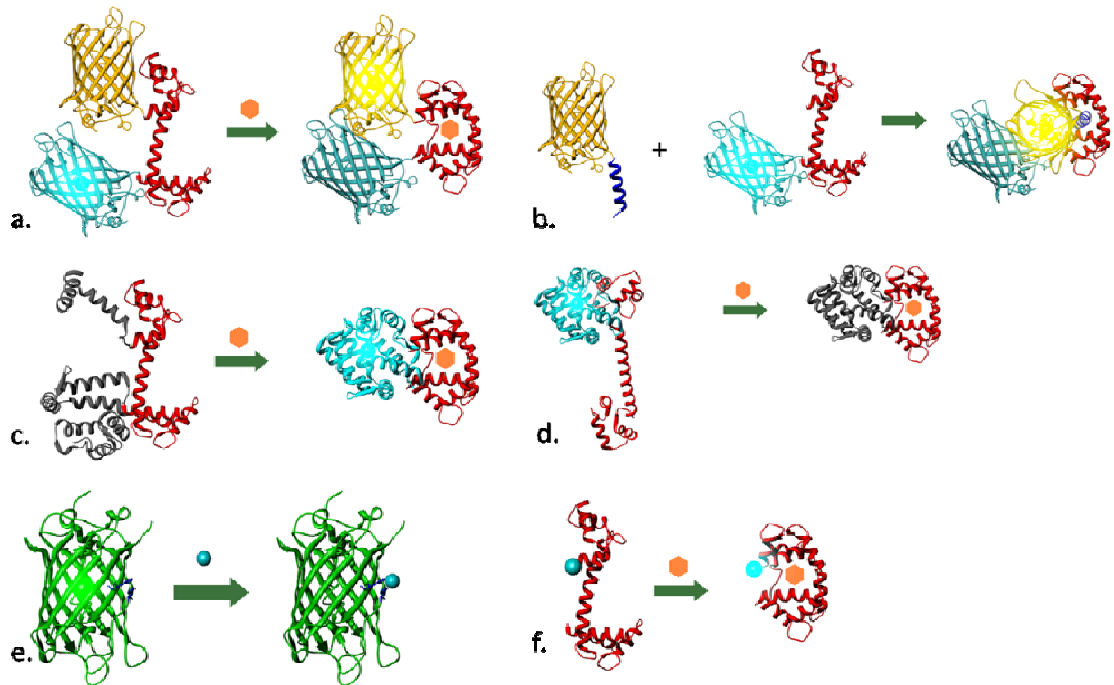


Figure 1.5 Types of genetically-encoded protein-based biosensors.

Strategies for the design of genetically-encoded biosensors include: intramolecular-FRET (a.), intermolecular-FRET (b.), reporter protein based “molecular switch” (c.), sensing protein based “molecular switch” (d.), reporter protein engineered as sensing protein (e.), and sensing protein engineered as reporter protein (f.).

increase in FRET. This approach is most applicable to studies of protein-protein interactions.[78] The third strategy is referred to as bimolecular complementation or simply as the “molecular switch” approach. In this design, a fluorescent or bioluminescent protein is rationally designed such that the amino acid sequence is split into two truncated fragments which, upon being placed within proximity of one another, can reassemble to form a functional reporter protein. Each fragment of the reporter molecule can then be fused to either end of a sensing protein such that upon analyte binding, the complimentary fragments are either pulled apart or brought together, resulting in a decrease or increase in signal, respectively.[72, 79] The fourth strategy is a variation of the third strategy in which truncated fragments of a sensing protein are placed at either end of an intact reporter protein. In this strategy, the binding event causes the sensing protein to reassemble which perturbs the structure of the reporter protein, thus resulting in a change in signal. In the fifth strategy, the reporter protein itself is engineered such that it displays a change in signal upon response to desired stimuli without the need for an exogenous sensing protein.[80-82] The sixth and final strategy is an extension of the fifth in which the sensing protein produces a measurable change in signal without the need for an exogenous reporter protein. Typically, this is accomplished by monitoring changes in intrinsic tryptophan fluorescence in a protein upon interaction with an analyte.[83, 84]

B. Chemically-modified Biosensing Systems

In contrast to genetically encoded protein-based biosensing systems, chemically-modified biosensors, as their name suggests, must be altered in some way after isolation of the sensing protein. Typically, this is accomplished by the covalent attachment of a small fluorescent organic molecule at a location within the protein structure which experiences a change in conformation upon ligand binding. As a result, the ligand-binding event causes a change in the microenvironment around the environmentally-sensitive fluorophore, resulting in a change in measured fluorescence intensity. Site-specific modification of proteins is most commonly accomplished by engineering the protein to contain a unique cysteine residue within the protein at the desired location and subsequent modification by a fluorophore using well-established sulfhydryl-reactive chemistry. The carefully-selected fluorophore can be sensitive to changes in pH, polarity, hydrophobicity, or other factors. As an alternative approach, a FRET-based strategy can be employed in which a sensing protein is modified with two fluorophores, a donor/acceptor pair, such that the ligand binding event alters the distance between the donor and acceptor resulting in a change in FRET. In this strategy, either the donor or acceptor can also be a genetically encoded reporter such as GFP or one of its variants.

The phenomenon of the alteration of fluorophore response in regards to its microenvironment is termed solvatochromism. These changes in fluorescence intensity can result from a number of factors including solvent polarity and viscosity, rate of solvent relaxation, probe conformational changes, rigidity of the local environment, internal charge transfer, proton transfer and excited state

reactions, probe-probe interactions, and changes in radiative and non-radiative decay rates.[85] When a fluorophore is bound to a location within a protein that undergoes a dramatic conformational change, one or more of the factors above may be altered, thus altering the spectral characteristics of the fluorescence emission (intensity, lifetime, wavelength maxima, etc). Because of the complexity of these processes, it is often difficult to determine the contribution of each of these factors to the change in the fluorescence that results from a change in protein conformation. Regardless, if this change in fluorescence emission follows a concentration-dependent response to the ligand, then this chemically-modified protein can be used for the development of a biosensing system.

Because these organic fluorophores are typically small molecules, this approach has the advantage of introducing a less pronounced disturbance in the sensing protein than the genetic fusion to a large reporter protein, thus there is a reduced chance of a detrimental effect on the activity of the sensing protein. Additionally, the variety of commercially-available fluorophores allows for the tailoring of spectral properties (wavelength, lifetime, quantum yield, etc) of the biosensing system. While some degree of customization is possible for genetically-encoded systems, the availability of mutants of reporter proteins with varying spectral properties is more limited.

SELECTION AND DESIGN OF MOLECULAR RECOGNITION ELEMENTS

Integral to the design of a protein-based sensing system is a molecular recognition element capable of accurately detecting the desired analyte with appropriate sensitivity and selectivity. As mentioned above, many types of proteins can be employed in the design of a protein-based sensing system. Among these are chemotaxis/transport proteins, antibodies, enzymes, regulatory proteins, etc. Each of these types of proteins has been employed in the development of biosensing systems using different strategies to take advantage of their unique characteristics and advantages.

A. Chemotaxis/transport Periplasmic Binding Proteins

Periplasmic binding proteins (PBPs) have been explored thoroughly as recognition elements for use in protein-based biosensing systems. PBPs function as transporters for their specific ligands across the periplasmic space. In general, members of this protein family selectively bind their respective target ligands with binding affinities in the 0.01-10 μM range.[86] Although there is great variety in their ligand specificity and amino acid sequence, these proteins typically have an overall conserved structural motif.[87] In general, PBPs consist of two globular domains each consisting of a β -sheet region surrounded by α -helices and connected by a short “hinge” region. (Figure 1.6) The region between the two domains forms a binding pocket in which the ligand binds, triggering an overall hinge-motion conformational change in the protein. PBPs and their derivatives have been identified for a wide-array of small molecules including sugars[72, 88-90], metal ions[90, 91], amino acids[92],

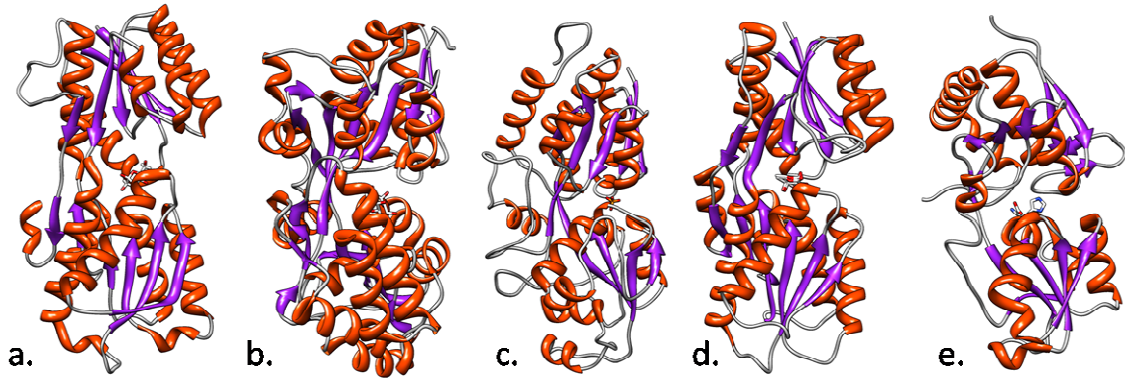


Figure 1.6 X-ray crystal structures of selected periplasmic binding proteins.

Periplasmic binding proteins from *E. coli* function as transporters/chemotaxis for a variety of small molecules across the periplasmic space. Proteins of this family consist of two lobes connected by a flexible hinge region and undergo a hinge-motion conformation change upon ligand binding. Examples are shown above: glucose/galactose binding protein (a.), maltose binding protein (b.), phosphate binding protein (c.), arabinose binding protein (d.), and histidine binding protein (e.).

sulfate/phosphate[90], etc. The diversity of PBPs, their inherent selectivity, their ease of expression and purification, and the customizability of their binding characteristics have contributed to the intense interest in recent years regarding their use as recognition elements in biosensing systems.

B. Antibodies

Antibodies, or immunoglobulins, are specialized proteins produced by the immune systems of animals to recognize a foreign target called an antigen. Generally, they are depicted as having a “Y”-shaped structure, with the antigen binding sites located at the end of each arm of the protein (Figure 1.7). In its most basic form, the antigen/antibody interaction can be thought of as a lock-and-key interaction. Antibodies are generally produced by injecting an animal with the antigen of interest, allowing time for the animal’s immune system to produce antibodies as part of a natural immune response, and then harvesting the appropriate antibodies by various methods. This well-defined procedure allows the production of antibodies with exceptional selectivity and affinity to a great variety of antigens ranging from small molecules to entire proteins and microorganisms.[93, 94]

Antibodies have been widely used as a molecular recognition element in numerous biotechnology and biosensing applications. Perhaps the most common and widely used application is that of enzyme-linked immunosorbent assays (ELISA). There are many variations of ELISA, however in the most basic

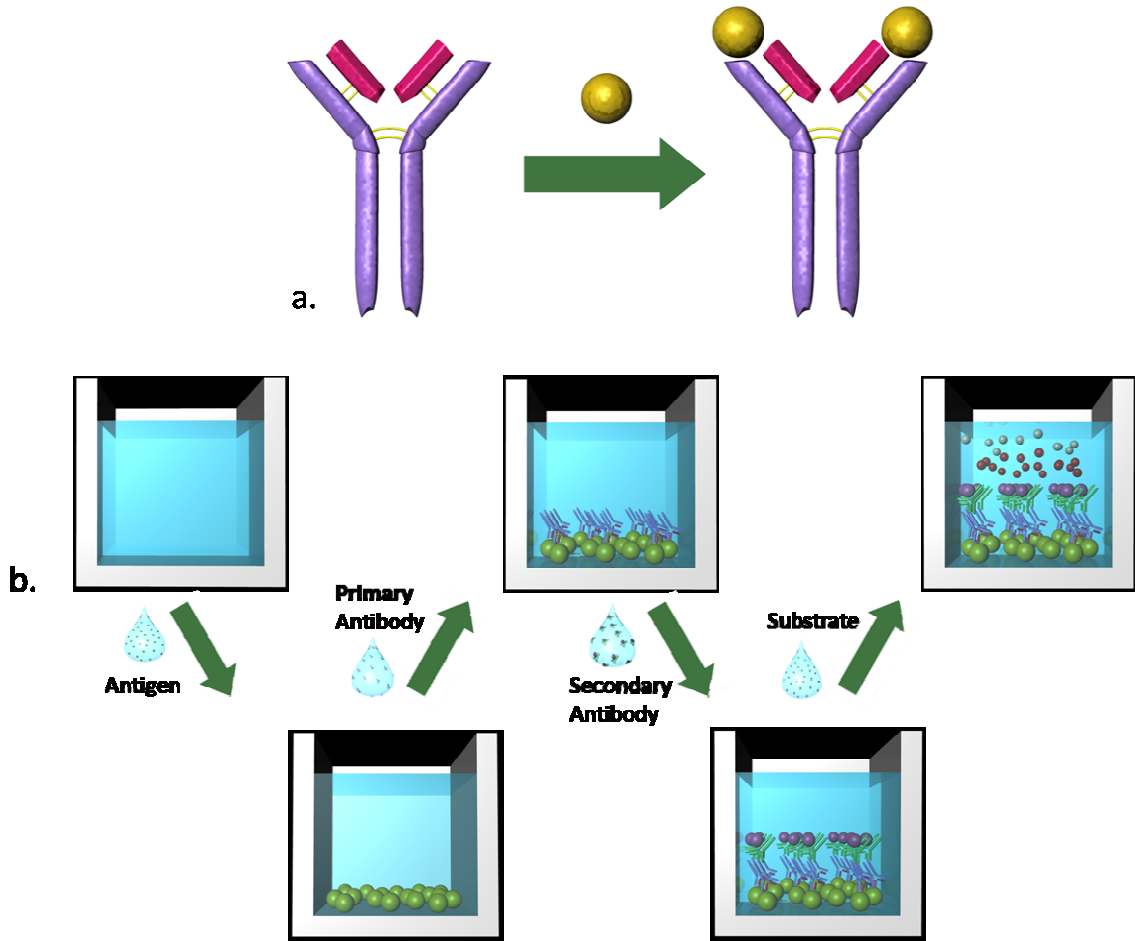


Figure 1.7 Antibody-based ELISA assay. In a typical antibody (a.), two antigen binding sites can be found facilitating the binding of an antigen molecule (yellow sphere). A very common antibody-based assay is called enzyme-linked immunosorbent assay (ELISA). In one common ELISA-based strategy, an antigen-containing solution is added to wells of a microtiter plate for immobilization. Following this, a primary antibody which recognizes the antigen is added. Next, a secondary antibody which has been conjugated to an enzyme is added which recognizes the primary antibody. Finally, a substrate for this enzyme is added, resulting in the production of a measurable signal. Between each steps, the microtiter plate wells are extensively washed.

strategy, a sample with an unknown amount of analyte/antigen is immobilized on a surface (usually a microtiter plate), and then the sample solution is removed and the surface is washed. Next, a solution containing a non-specific protein, typically bovine serum albumin, is added to coat the exposed surface not covered by the sample. Then a primary antibody with an antigen binding site for the analyte is added and allowed to bind to the analyte on the surface. A secondary antibody, with an enzyme attached to it, is added next, which recognizes the primary antibody and binds to it. Finally, an appropriate substrate for the attached enzyme is added in order to produce an observable signal which corresponds to the concentration of analyte/antigen present in the initial sample. Most commonly, an enzyme-substrate pair is chosen which results in a colorimetric signal. However, alternative approaches employing bioluminescent photoprotein such as aequorin[19], fluorescent proteins such as GFP and its variants[95] or fluorophore-labeled antibodies also exist.

C. Enzymes

Enzymes are a class of proteins which catalyze biochemical reactions by reducing the activation energy. Within the active site of an enzyme, a substrate binds and is converted to a product. In complex biological systems, enzymes must have exceptional specificity towards their appropriate substrate and an affinity to their substrate at biologically relevant concentrations. These characteristics make enzymes amenable for use in the development of biosensing systems. Indeed, perhaps the most commercially successful

example of a protein-based biosensing system, the personal blood glucose meter, is an enzyme-based system usually employing the enzyme glucose oxidase.[96] The current generation of glucose meters are electrochemical-based sensors which employ glucose oxidase and a mediator coupled to an electrode for electrochemical measurement.

In addition to electrochemical approaches, fluorescence-based approaches are also being pursued in the development of enzyme-based biosensors. Enzyme activity is also often associated with a significant conformational change which can be utilized in the development of a chemically-modified biosensor. This has been used in the development of a biosensor for glucose based on the enzyme glucokinase by site-specific attachment of a fluorophore.[97] Glucokinase functions by transferring phosphate from ATP to the C6 position on glucose. During this enzymatic reaction, a large conformational change occurs. This conformational change can be observed as a change in fluorescence when the enzyme is modified with an appropriate fluorophore. In addition, some enzymes require the binding of a cofactor in addition to a substrate. In the case of glucose oxidase, the cofactor flavin adenine dinucleotide which is fluorescent and displays a small change in fluorescence upon glucose binding.[98]

D. Regulatory Proteins

Regulatory proteins, as discussed in some detail above, can also be used in the development of a protein-based biosensing system. Regulatory proteins

are typically quite specific to their effector molecule. Regulatory proteins, in both positively and negatively regulated systems, typically undergo significant conformational changes upon binding their effector molecules. Through the strategies described previously, these conformational changes can be followed when coupled with either a reporter protein fusion in a genetically-encoded strategy, or a site-specifically attached fluorophore in a chemically-modified approach. In either case, the resulting conformational change results in a change in fluorescence signal intensity which can be correlated to the concentration of the effector/analyte molecule. In one such system, the protein MerR, which binds specifically to Hg^{2+} has been engineered such that upon binding, a change in fluorescence is observed.[99] Another example of a regulatory protein-based sensing system is the detection of cGMP by fusing the regulatory element of various cGMP protein kinases to variants of GFP.[100]

CONCLUSIONS AND FUTURE POTENTIAL

Continued work in the fields of cell-and protein-based biosensors should contribute to further exploitation and enhancement of the unique advantages that they offer in the detection of a variety of analytes in different types of samples using diverse analytical platforms. Cell-based biosensing systems are most promising in their applicability to the development of rugged biosensing systems which give some degree of bioavailability information on the target analyte. The ruggedness of cell-based systems will only increase with the further development of spore-based biosensing systems, which is a growing field. Protein-based

sensing systems, due to their rapid assay times, are the best candidate for the development of real-time monitoring devices which are especially useful in therapeutic and biomedical devices.

The identification of additional regulatory proteins, receptors, enzymes, etc. should expand the ranks of chemical species that can be detected. Moreover, the identification, characterization, and alteration of new light-emitting bioreporters should lead to advanced, multiplexed assays capable of measuring several analytes simultaneously. Advancements in optical instrumentation and miniaturization should, undoubtedly, yield smaller, more rugged, less expensive methods along with the selectivity and sensitivity afforded by these genetically engineered cells. Miniaturization to array-based or microfluidic chip-based platforms reduces volumes of reagents and samples as well as wastes produced. There is no doubt that the future is bright for cell- and protein-based bioluminescent biosensors as they will find further application in the fields of environmental monitoring, toxicology, pharmacology, drug-screening, and medical/clinical applications.

STATEMENT OF RESEARCH

The underlying theme of the work presented here is to engineer bacterial cells and proteins to improve their response characteristics such that they can be integrated into biosensing systems. Nature has devoted billions of years to evolving the diversity of biological functionalities, which is evident in the variety of organisms that today inhabit all of the environments of Earth ranging from rainforests to water reservoirs beneath the South Pole to volcanic vents on the ocean floor to boiling hot, acidic mineral springs. Biological moieties (i.e., proteins, enzymes, tissues, and whole cells) have developed mechanisms to detect and respond to very specific chemical species in complex mixtures as well as tolerate and thrive in harsh living environments. Here, we aim to take advantage of the exceptional inherent selectivity, sensitivity, and stability that exists throughout the natural world in the development of analytical systems to address challenges regarding the detection of both environmentally- and physiologically-relevant small molecules. The work presented in this dissertation was guided by the following hypotheses:

- Regulatory genetic circuits from a microorganism that respond to a specific small molecule effector can be exploited by replacing the genes regulated in the native organism with an appropriate reporter gene, thus allowing detection and quantification of the effector molecule to which the regulatory system responds to.

- By isolating and rationally engineering appropriate protein (i.e., hinge-motion periplasmic chemotaxis proteins and regulatory proteins) that serve as the biorecognition element in a biosensing system, we can attain the required selectivity and sensitivity needed to detect small molecules of interest in complex sample matrices.
- Utilizing a protein from a hyperthermophile, *Thermotoga maritima*, we can develop a protein-based sensing system with remarkable thermal stability. This increased stability results in a sensing system that is rugged enough to operate at physiological temperatures for extended periods of time. Additionally, the improved stability makes this sensing system amenable to storage and transport in extreme environments.

The work presented here describes research that sets out to explore the hypotheses set forth above. Biosensing systems have been developed based upon both intact living cells, as well as isolated regulatory and binding proteins. Through each of these projects, we set out to maintain the desirable characteristics inherent in the native biological moieties while rationally engineering them as appropriate to tailor other characteristics (i.e., binding affinity, thermal stability, and selectivity) when appropriate. Below is a brief description of the chapters to follow:

- Chapter two describes the development of a whole-cell sensing system for the detection of hydroxylated polychlorinated biphenyls (OH-PCBs). The regulatory protein (HbpR) and promoter (P_{hbpCAD}) from the organism

Pseudomonas azelaica were engineered to regulate the expression of the bioluminescent reporter protein, bacterial luciferase (LuxAB). The result is a bacterial cell that produces bioluminescence whose intensity can be correlated to the concentration of OH-PCBs that are present in environmental or biological samples.

- Chapter three details the development of a protein-based sensing system based upon the aforementioned HbpR regulatory protein. This protein undergoes a drastic conformational change upon effector molecule binding. By labeling the native cysteines present in the protein with an environmentally-sensitive fluorophore, we were able to observe a concentration-dependent change in fluorescence in response to the concentration of OH-PCBs present.
- In chapter four, the development of a protein-based sensing system for glucose based upon the periplasmic glucose binding protein (GBP) from *E. coli* is described. Protein-based sensing systems using GBP labeled with an environmentally-sensitive fluorophore have been previously developed with binding affinities in the micromolar range. In order to shift the binding affinity into the physiologically-relevant millimolar range, truncated fragments of native GBP (tGRPs) were designed and characterized.
- The design and characterization of a protein-based sensing system for glucose based upon GBP from the hyperthermophile *Thermotoga*

maritima is described in chapter five. The biosensing system presented here differs from those presented in the previous chapters, in that the source of fluorescence measured is the protein's intrinsic tryptophan fluorescence. This eliminates the need for an extra chemical labeling step. Additionally, by employing a binding protein from a hyperthermophile, exceptional thermal stability has been observed, resulting in a remarkably rugged biosensing system.

CHAPTER TWO

HYDROXYLATED POLYCHLORINATED BIPHENYLS DETECTION BASED ON A GENETICALLY ENGINEERED BIOLUMINESCENT WHOLE-CELL SENSING SYSTEM

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INTRODUCTION

Since the 1970s, the apparent toxicities of polychlorinated biphenyls (PCBs) have been scrutinized in much detail. PCBs have been shown in numerous studies to contribute to negative health effects[101-104] and to be persistent in biological and environmental samples.[105] Hydroxylated PCBs (OH-PCBs) have also been more closely examined as potentially exhibiting significant toxic health effects. OH-PCBs have been detected in the environment at concentrations as much as 3.5 times that of PCBs, possibly originating from sewage treatment plants and byproducts of industrial-scale reactions involving biphenyl and biphenylol.[106] Additionally, OH-PCBs are also present in biological fluids as metabolites of PCBs.[105] Many OH-PCBs have been identified in human serum samples and may be present at concentrations comparable to the parent PCBs.[101] It has been shown that some OH-PCBs have estrogenic and antiestrogenic activities in various mammalian models,[101-104] inhibit gap junctional intercellular communication,[103] activate aryl

hydrocarbon receptors,[103] and form DNA adducts leading to damage of DNA.[107]

Due to the toxicity and environmental and biological persistence of OH-PCBs, it is necessary to have efficient and economical methods to detect and quantify them. Currently, the methods of choice are GC-MS and LC-MS.[108] These methods have excellent detection limits and can effectively identify and quantify OH-PCBs. However, sample extraction, cleanup and derivatization steps are required for analysis. These factors combined with the expenses associated with instrumentation and the need for trained laboratory technicians quickly drives up the cost and time required for analysis.

Alternatively, whole-cell sensing systems provide several advantages over traditional techniques. Most notably, the speed of analysis is increased due to the lack of need for extensive sample preparation steps and the ability to evaluate multiple samples in one analytical run. Whole-cell sensors, due to the inherent selectivity of the recognition/regulatory proteins involved, can be used in complex sample matrices without significant impact from interferants. In addition, a whole-cell sensing system could be developed into a field-portable assay and used as an on-site screening tool for both environmental and biological samples, allowing for a more effective selection of samples to be evaluated in more detail. Lastly, information on bioavailability of analytes is obtained with whole-cell sensing systems, which facilitates the prediction of fate and effect of the pollutants for toxicological studies.

A common strategy in the development of a whole-cell sensing system is placing a gene which encodes for a reporter protein under the control of a specific recognition element, such as a regulatory protein, for the analyte of interest as shown in Figure 2.1. Biosensors of this type have been developed for a variety of analytes. More in-depth information and examples can be found in a number of reviews.[109-111] Specifically, we have developed a whole-cell sensing system for the detection of OH-PCBs by employing the strain *Pseudomonas azelaica* HBP1. This bacterium contains the *hbpCAD* genes, which are responsible for the degradation of hydroxylated biphenyls. The expression of these genes is negatively regulated by a regulatory protein encoded by the gene *hbpR* located upstream from the *hbpCAD* genes. A strain of *Escherichia coli* carrying a recombinant plasmid consisting of the *luxAB* reporter gene, coding for bacterial luciferase, under control of the HbpR regulatory protein was constructed[112] and employed as a whole-cell sensing system in the present study. In the presence of analytes, such as OH-PCBs, the regulatory protein HbpR activates transcription through the *hbp* promoter resulting in the expression of the reporter gene. This expression can then be monitored by measuring bioluminescence emission after addition of decanal, a substrate for luciferase. Within a certain range of analyte concentrations, the expression of the reporter gene is dose-dependent, therefore, the intensity of the analytical signal is directly related to the amount of target compound.

Analyzing the concentration of OH-PCBs in serum samples as a biomarker of PCB exposure is essential for toxicological studies and remediation

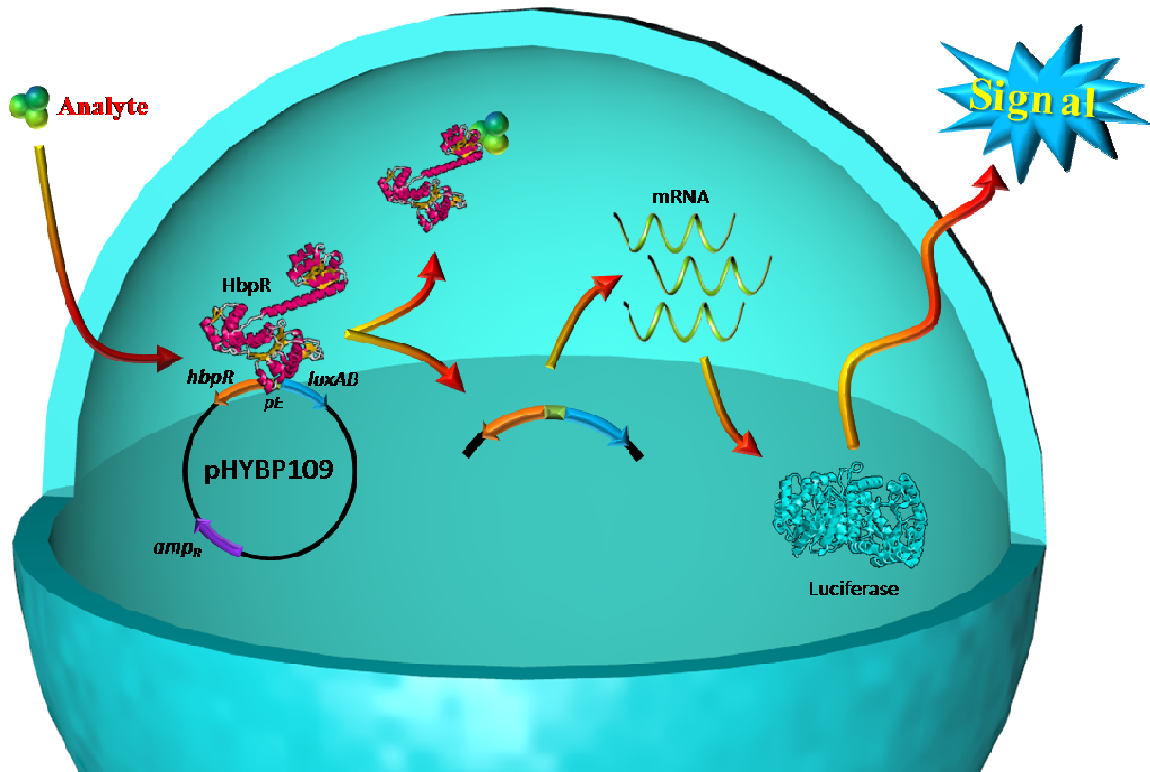


Figure 2.1. Schematic of Genetically Engineered Bioluminescent Whole-Cell Sensing System. The analyte diffuses into the cell where it binds the regulatory protein HbpR which is bound to the reporter plasmid, pHYBP109. Upon binding, HbpR undergoes a conformational change, releases from the reporter plasmid, and allows expression of the reporter protein, LuxAB which generates a bioluminescent signal.

purposes. The hydrophilic nature of the hydroxyl group in OH-PCBs suggests that these chemicals may be readily excreted from the body. However, the most predominant PCB metabolites found in biological fluids contain between five to seven chlorines with one hydroxyl group in the biphenyl ring.[113, 114] Therefore, the possibility of retention rather than excretion also exists due to the increased hydrophobicity contributed by physicochemical properties of chlorines attached to the biphenyl backbone. Moreover, their high lipophilicity and affinity to certain proteins such as the thyroxin-transporting protein, transthyretin (TTR) lead to the retention of OH-PCBs in different body compartments, mainly in blood.[115]

In this study, we have employed a genetically engineered bacterium to develop a whole-cell sensing system to detect the presence of a variety of OH-PCBs in both environmental and biological samples. This sensing system has been optimized with respect to important assay conditions, and the feasibility of the application of this system as a screening tool has been discussed.

MATERIALS AND METHODS

Chemicals and media

2-Hydroxybiphenyl was obtained from Sigma-Aldrich Corp. (St. Louis, MO). In Table 2.1, OH-PCBs No 2, 5, 6, and 8-12 were obtained from Accustandard Inc. (New Haven, CT), OH-PCBs No 3 and 4 were obtained from

Table 2.1. Response of the whole-cell sensing system to various OH-PCBs.

No	Compound	$\log (A/A_0)^a$	$-\log ED_{50}^b$	Detection Limit (M) ^c
1	2-Hydroxy-4'-chlorobiphenyl	0.44	1.76 ± 0.37	1.0×10 ⁻⁶
2	2-Hydroxy-3'4'-dichlorobiphenyl	0.88	5.66 ± 0.07	5.0×10 ⁻⁷
3	2-Hydroxy-3,5-dichlorobiphenyl	0.50	2.09 ± 0.24	5.0×10 ⁻⁵
4	2-Hydroxy-2',3,5'-trichlorobiphenyl	0.56	3.86 ± 0.25	1.0×10 ⁻⁵
5	2-Hydroxy-2',4',6'-trichlorobiphenyl	0.37	3.21 ± 0.06	1.0×10 ⁻⁸
6	2-Hydroxy-2',5,5'-trichlorobiphenyl	0.49	4.17 ± 0.03	5.0×10 ⁻⁶
7	2-Hydroxy-3',4',5-trichlorobiphenyl	0.24	4.84 ± 0.15	1.0×10 ⁻⁹
8	2-Hydroxy-3',5,5'-trichlorobiphenyl	0.22	5.16 ± 0.32	1.0×10 ⁻⁷
9	2-Hydroxy-2',3',4',5'-tetrachlorobiphenyl	0.47	3.21 ± 0.06	5.0×10 ⁻⁵
10	2-Hydroxy-2',3',5',6'-tetrachlorobiphenyl	0.48	4.47 ± 0.11	5.0×10 ⁻⁶
11	2-Hydroxy-2',4',5,6'-tetrachlorobiphenyl	0.20	3.36 ± 0.62	1.0×10 ⁻⁶
12	2-Hydroxy-2',3',4',5,5'-pentachlorobiphenyl	0.35	4.35 ± 0.76	1.0×10 ⁻⁶
13	3-Hydroxy-4'-chlorobiphenyl	0.46	2.29 ± 0.24	1.0×10 ⁻⁷
14	4-Hydroxy-4'-chlorobiphenyl	0.31	2.87 ± 0.19	5.0×10 ⁻⁵
15	4-Hydroxy-2',3-dichlorobiphenyl	0.75	4.43 ± 0.09	1.0×10 ⁻⁸
16	4-Hydroxy-2',5'-dichlorobiphenyl	0.61	3.46 ± 0.07	1.0×10 ⁻⁷
17	4-Hydroxy-3,3'-dichlorobiphenyl	0.64	4.58 ± 0.14	1.0×10 ⁻⁸
18	4-Hydroxy-3,4'-dichlorobiphenyl	0.60	3.75 ± 0.05	1.0×10 ⁻⁶
19	4-Hydroxy-3',4'-dichlorobiphenyl	0.41	3.49 ± 0.69	1.0×10 ⁻⁸
20	4-Hydroxy-3,5-dichlorobiphenyl	0.63	3.49 ± 0.05	5.0×10 ⁻⁷
21	4-Hydroxy-2',3,5-trichlorobiphenyl	0.32	3.75 ± 0.05	1.0×10 ⁻⁶
22	4-Hydroxy-3,3',4'-trichlorobiphenyl	0.82	4.79 ± 0.05	1.0×10 ⁻⁷
23	4-Hydroxy-3,3',5-trichlorobiphenyl	0.42	2.49 ± 0.11	1.0×10 ⁻⁶
24	4-Hydroxy-3,3',5'-trichlorobiphenyl	0.50	4.21 ± 0.06	1.0×10 ⁻⁶
25	4-Hydroxy-3,4',5-trichlorobiphenyl	0.38	3.40 ± 0.14	1.0×10 ⁻⁶
26	4-Hydroxy-3,3',4',5-tetrachlorobiphenyl	0.21	3.49 ± 0.30	1.0×10 ⁻⁵
27	4-Hydroxy-2',3,4',5-tetrachlorobiphenyl	0.12	4.42 ± 0.26	1.0×10 ⁻⁷

^a The response ratio (A/A_0) was calculated by dividing the maximum bioluminescence signals for each hydroxylated PCB (A) by the bioluminescence signals for the blank (A_0)

^b ED_{50} was defined as concentration at 50% maximum induction, and was calculated by using GraphPad Prism 4.0 software.

^c Detection limit was defined as the analyte concentration that corresponds to a signal-to-noise ratio of 3.

Ultra Scientific Inc. (North Kingstown, RI), and OH-PCBs No 1, 7, and 13-27 were kind gifts from Hans Lehmler at the University of Iowa. The purities (\geq 95.0%) of these synthesized OH-PCBs were verified by Agilent GC-MS 5975 prior to experiments.

Dimethyl sulfoxide (DMSO, \geq 99.9%, for molecular biology), n-decanal (\geq 99%, for GC, liquid), ethanol (anhydrous, \geq 99.5%, 200 proof), glycerin (meets USP testing specifications), and human serum (from clotted human male whole blood, sterile-filtered, mycoplasma tested, virus tested) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Deionized distilled water was produced by a Milli-Q water purification system (Millipore, Bedford, MA).

Escherichia coli strains were grown at 37 °C on Luria-Bertani (LB) medium (BIO 101, Vista, CA) containing ampicillin (100 μ g/mL, Sigma-Aldrich Corp., St. Louis, MO). For the luciferase induction assay, mineral medium (MM) defined by Gerhardt *et al.*, [116] was prepared and supplemented with 0.01% tryptone, 0.005% yeast extract, and 10 mM D-(+)-glucose before use, as described by Jaspers *et al.* [112] MM (per liter) was prepared from the ingredients: 1.00 g of NH_4Cl , 3.49 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.77 g of KH_2PO_4 , 20 mL of Hunter's vitamin-free mineral base, and 2.0 mL of a vitamin solution, adjusted to pH 6.8. Hunter's vitamin-free mineral base contained (per liter): 10 g of nitrilotriacetic acid (neutralize with 6.00 g of KOH), 14.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.33 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 9.74 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 99 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 50 mL of the Metals 44 solution. The composition of Metals 44 (per 100 mL) was as follows: 387 mg

of $\text{Na}_4\text{EDTA}\cdot 4\text{H}_2\text{O}$, 1.10 g of $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 914 mg of $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 154 mg of $\text{MnSO}_4\cdot \text{H}_2\text{O}$, 39.2 mg of $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 24.8 mg of $\text{Co}(\text{NO}_3)_2\cdot 6\text{H}_2\text{O}$, 17.7 mg of $\text{Na}_2\text{B}_4\text{O}_7\cdot 19\text{H}_2\text{O}$, and was neutralized with H_2SO_4 . The vitamin solution (per 100 mL) was prepared by mixing 0.50 mg of biotin, 50 mg of nicotinic acid, and 25 mg of thiamine hydrochloride. MM was sterilized and stored at 4 °C before use. All chemicals for MM preparation were purchased from Sigma-Aldrich Corp. (St. Louis, MO), and were at least cell culture tested grade.

Preparation of E. coli cells harboring plasmid pHYBP109

Cells were obtained as a frozen glycerol stock. A stab was taken from this stock and grown overnight at 37 °C in a culture tube containing 2 mL of LB medium and ampicillin (100 µg/mL). The following day, the 2 mL culture was transferred to a 1 L flask containing 250 mL of LB medium and grown at 37 °C to an optical density at 600 nm (OD_{600}) of approximately 0.90. The cells were then dispensed into 1.5 mL microcentrifuge tubes in 1 mL aliquots and stored at -80 °C until use. A volume of 50 µL of sterilized glycerin was added to each 1 mL stock of cells for preservation purposes. Unless otherwise specified, all the cells were grown in a rotary shaker at 200 rpm.

Detection of compounds in dimethyl sulfoxide

Stock solutions of each compound were prepared at a concentration of 1×10^{-2} M by dissolving the appropriate mass of each compound in 1.0 mL of DMSO in a microcentrifuge tube and vortexing until dissolved. From these, solutions in a concentration range of 5.0×10^{-3} - 1.0×10^{-9} M were prepared by serial dilution of the original stock solution in DMSO. In addition, a blank was prepared containing only DMSO. Each set of solutions was prepared fresh and the 1×10^{-2} M stock solutions were saved and frozen at -20 °C after use.

Immediately prior to use in each assay, a microcentrifuge tube containing a 1 mL aliquot of cells was removed from the -80 °C freezer and placed in a room temperature water bath for 2 minutes, and then on ice. For the final assay solution, 1.9 mL of MM, 33 μ L of thawed cell suspension, and 20 μ L of a compound at each concentration along with a blank as prepared above were dispensed into a series of 14 mL culture tubes in triplicate. These tubes were placed at 30 °C on an orbital shaker at 225rpm for a 4 hour incubation period.

Following incubation, a volume of 200 μ L of the cell suspension from each culture tube was dispensed into the wells of a 96-well microtiter plate in triplicate. A volume of 100 μ L of stock n-decanal substrate solution (2 mM in 1:1 ethanol/ H_2O) was added into the reaction assay by automated injection for the bioluminescence signal measurement. Light output was integrated from 5 s to 15 s after n-decanal injection for each of the wells in the plate using a POLARstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany). The total light signal was expressed as relative light units (RLU).

For experimental optimization, the general protocol described above was employed, unless otherwise specified in the text. All assays were conducted in triplicate.

Detection of model compound in human serum

2-Hydroxy-3',4'-dichlorobiphenyl was used to evaluate the assay in human serum. This compound was prepared as above in a concentration range from 1×10^{-2} M – 1×10^{-8} M in DMSO along with a blank containing only DMSO. In a separate set of microcentrifuge tubes, 10 μ L of each of these solutions was dispensed into 90 μ L of human serum. This resulted in serum samples with the compound of interest present in a range of 1×10^{-3} M – 1×10^{-9} M along with a blank. Additionally, a set of samples was prepared using deionized water instead of human serum as a comparison. These solutions were then used to complete the assay as described above.

Detection of a compound mixture in human serum

A mixture of the following 10 OH-PCBs was prepared at a concentration of 1×10^{-3} M with respect to each individual compound in DMSO: 2-hydroxy-3',4'-dichlorobiphenyl, 4-hydroxy-3,3',4'-trichlorobiphenyl, 2-hydroxy-3',4',5-trichlorobiphenyl, 2-hydroxy-3',5,5'-trichlorobiphenyl, 3-hydroxy-4'-chlorobiphenyl, 4-hydroxy-2',3,4',5-tetrachlorobiphenyl, 4-hydroxy-2',5'-dichlorobiphenyl, 4-

hydroxy-3',4'-dichlorobiphenyl, 4-hydroxy-3,3'-dichlorobiphenyl, 4-hydroxy-2',3-dichlorobiphenyl. To prepare this solution, 10 μL of the 1.0×10^{-2} M stock solution of each compound was dispensed into a single microcentrifuge tube. Serial dilutions were made using this solution to create a mixture in DMSO in a concentration range of 1×10^{-3} – 1×10^{-8} M with respect to each compound and a concentration of 1×10^{-2} M – 1×10^{-7} M with respect to total OH-PCBs. In a separate set of microcentrifuge tubes, 10 μL of each of these solutions was dispensed into 90 μL of human serum, yielding samples of the mixture in human serum in a concentration range of 1×10^{-4} – 1×10^{-9} M with respect to each compound. These samples were then used to complete the assay as described above.

Statistical analysis

The results presented are the averages of the values obtained in three independent experiments. GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) was used to generate non-linear best-fit lines of the data.

RESULTS AND DISCUSSION

The strain *Pseudomonas azelaica* HBP1 is able to use hydroxylated biphenyls as sole carbon and energy sources. The mechanism for the degradation of 2-hydroxybiphenyl by *P. azelaica* HBP1 has been well

characterized.[117] The metabolism involves three enzymes encoded by the *hbpCAD* genes, which are negatively regulated by the HbpR protein. To study the P_{hbpC} promoter activity of *P. azelaica* HBP1, a plasmid (designated as pHYBP109) containing *hbpR-P_{hbpC}-luxAB* fusion was constructed and transformed to *E. coli* by Jaspers *et al.*[112] Induction experiments were carried out using 2-hydroxybiphenyl. Increasing bioluminescence signals upon induction with 0.2 mM of 2-hydroxybiphenyl for increasing induction times suggested the direct activation of P_{hbpC} promoter by the compound binding to the HbpR protein.[112] In our laboratory, initial experiments were conducted by treating these recombinant *E. coli* cells with varying concentrations of 2-hydroxybiphenyl. A preliminary detection limit of 1×10^{-6} M could be obtained (data not shown), under the experimental conditions reported by Jaspers *et al.*[112] The detection limit is defined as the minimum analyte concentration that corresponds to a signal-to-noise ratio of 3.

A whole-cell sensing system is a genetically modified system constructed in such a way that in the presence of an analyte, expression of a reporter protein is triggered. In the plasmid used in this study, *hbpR*, the gene encoding for the HbpR protein, is positioned upstream from the *luxAB* genes, which code for the bioluminescent reporter protein, luciferase. In this way, the expression of luciferase is placed under the control of the P_{hbpC} promoter, which is regulated by the HbpR protein. Thus, the expression of luciferase in *E. coli* harboring pHYBP109 is mediated by the HbpR regulatory protein from the same regulatory circuit. In the absence of hydroxylated biphenyls, the HbpR protein binds to the

promoter and prevents the expression of *luxAB* genes. In the presence of hydroxylated biphenyls, the HbpR protein undergoes a conformational change upon binding of the inducer molecules, causing release of the protein-inducer complex from the promoter. As a result, expression of the reporter protein occurs.

In a study carried out by Jaspers *et al.* to evaluate the selectivity of the HbpR protein, a group of chemicals such as biphenylic compounds, polycyclic aromatic hydrocarbons, and monoaromatic hydrocarbons, among others, were screened using engineered bacteria.[112] It was found that only a few structurally similar chemicals containing the biphenyl backbone showed significant activity. In fact, when the signals were normalized to the response to 2-hydroxybiphenyl at a concentration of 2 mM, relative luciferase activities more than 32% were observed for only a narrow range of compounds. Additionally, some chemicals showing activity had a hydroxyl group at the ortho biphenyl ring position. On the other hand, biphenyl, chlorobiphenyl, or compounds with a monoaromatic structure failed to show activity. Because of the selectivity of the HbpR protein in recognizing compounds with biphenyl backbone and a hydroxyl group, we envisioned that employing engineered *E. coli* harboring plasmid pHYBP109 as a whole-cell sensing system would permit us to detect a group of structurally similar OH-PCBs. Moreover, the broad inducer range of TbuT[118] and XylR[119], which belong to the same NtRC family of proteins as HbpR, further supported the investigation of the inducer spectrum of HbpR protein by exposing the recombinant cells to OH-PCBs. As a pioneer study, cells were exposed to

five randomly selected OH-PCBs. Significant increase of the bioluminescence signal as compared to a blank was observed for all five compounds tested. The maximum signal ratio of 1.72 ± 0.11 (mean \pm standard deviation; $n=3$) was found by using 2-hydroxy-3',4'-dichlorobiphenyl at a concentration of 1×10^{-3} M after 2h incubation period. Thus, this chemical was used as a model compound for further optimization of assay parameters.

Because only the regulator and promoter region of the *hbp* operon was inserted into the luciferase expression vector, the degradation of OH-PCBs by the cells is unlikely. For this reason, the toxicity of OH-PCBs to the bacteria used in our assay was evaluated. Cell growth was monitored with/without the addition of 2-hydroxy-3',4'-dichlorobiphenyl, at a final concentration of 1×10^{-5} M, to the medium containing cells harboring the pHYBP109 plasmid. Optical densities at 600 nm (OD_{600}) were checked every hour for 8 hours. Observed differences in OD_{600} of less than 10% between the two cell growth curves indicate that the toxicity of the compound is negligible at the tested concentration.

Important assay parameters were optimized in order to improve the overall performance of the biosensing system. Among these were the growth stage at which the cells were harvested, the storage method of the harvested cells, and the length and temperature of the incubation of the cells with the analytes. The optimum biosensing conditions were found to be harvesting cells at an optical density of approximately 0.9 measured at 600 nm, storage of the cells at -80 °C until use, and incubating the cells at 30 °C for 4 h during the assay.

Once all biosensing system parameters were optimized, the genetically engineered whole cells were exposed to varying concentrations of 2-hydroxy-3',4'-dichlorobiphenyl. An increase in the bioluminescence signal was observed with increasing concentration of the compound as shown in Figure 2.2. Data points were fitted with a sigmoidal dose-response curve, and a correlation coefficient R^2 of 0.9977 was obtained. The detection limit falls in the order of 10^{-8} M. When the analyte concentration was in the range of 1×10^{-7} M to 1×10^{-3} M, a rapid increase of the bioluminescence signal was observed. Concentrations of the model compound higher than 1×10^{-3} M caused the bioluminescence signal to decrease significantly (data not shown). This observation is in agreement with the data published by Hay *et al.* for the detection of 2,4-dichlorophenol using a bioluminescent whole-cell reporter.[120] It is speculated that at higher concentrations of the analyte, there is a toxic effect, which leads to a reduced number of living cells and a subsequent significant decrease in the response.

The next step in our research aimed at evaluating the response of the whole-cell sensor to a variety of OH-PCBs. However, it is difficult to assess the most predominant OH-PCBs in human serum[104] because of the unavailability of these compounds for use as standards from commercial sources. For that, a range of 27 commercially available OH-PCBs at various concentrations were tested. A dose-dependent response was observed for all of these compounds. A representative example for 4-hydroxy-3,3',4'-trichlorobiphenyl is reported in Figure 2.2. The data from each analyte was fit with a sigmoidal dose-response curve, and parameters such as $\log ED_{50}$ and $\log (A/A_0)$ were calculated and

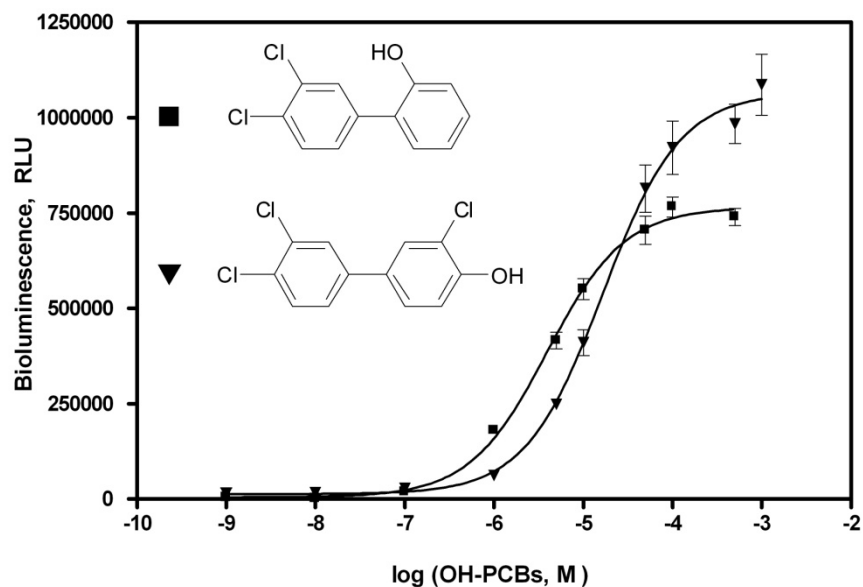


Figure 2.2. Dose-response curves of the whole-cell sensing system. The curves were obtained with 2-hydroxy-3',4'-dichlorobiphenyl (■) and 4-hydroxy-3,3',4'-trichlorobiphenyl (▼) under optimized experimental conditions. The bioluminescence signals have been corrected with respect to the blank. Values represent the means \pm standard deviation of triplicate determinations.

summarized in Table 1. ED_{50} was defined as the concentration at 50% of maximum induction, and was calculated by using GraphPad Prism 4.0 software. The induction ratio (A/A_0) was calculated by dividing the maximum bioluminescence signal for each hydroxylated PCB (A) by the bioluminescence signal for the blank (A_0). The maximum induction levels and the concentrations to achieve the maximum induction varied among the analytes tested. The dynamic range for the detection of hydroxylated PCBs covered two to five orders of magnitude. The detection limits ranged from 1×10^{-9} to 1×10^{-5} M. These results are in agreement with previous studies which demonstrate differential light responses in whole-cell sensing systems to various members of a class of related compounds.[121, 122] An attempt to explain the differences in the responses to the chemicals, based on their molecular structure, is ongoing at this stage.

In order to validate the response of this luciferase-based whole-cell sensing system to the PCB metabolites in a biological matrix, human serum was spiked with a range of concentrations of the model compound. The assay was carried out under the optimized experimental conditions. As shown in Figure 2.3, a dose-response curve was obtained with a $\log ED_{50}$ of -5.29, and a detection limit of 5.0×10^{-8} M. Increased bioluminescence signals were observed for the serum samples spiked with 2-hydroxy-3',4'-dichlorobiphenyl, as compared to the signals produced by the same analyte concentrations in the absence of the biological matrix. It is believed that this increased signal is due to the presence of proteins found in serum. These proteins may prevent the analyte from

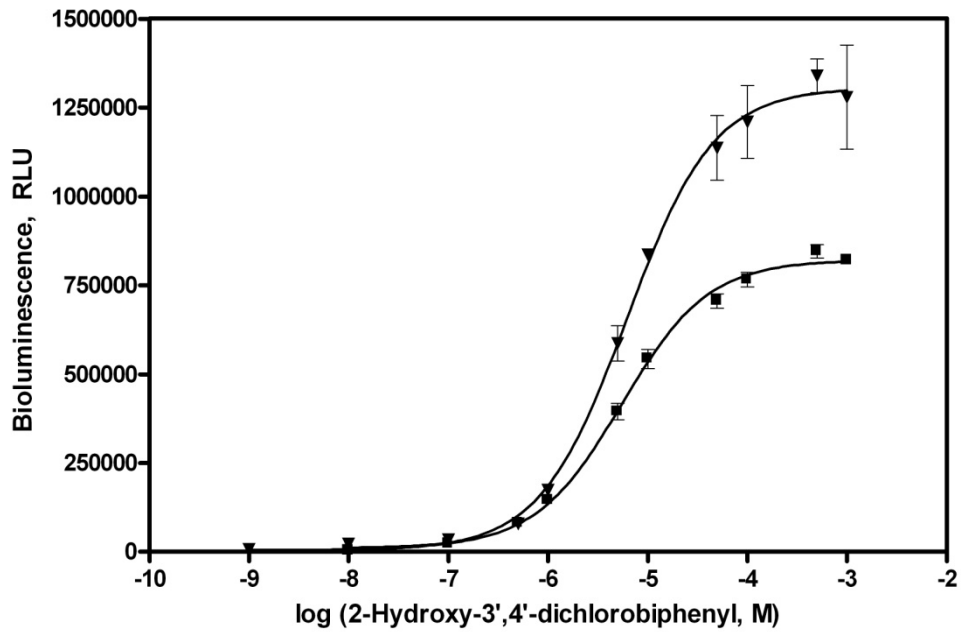


Figure 2.3. Dose-response curves of the whole-cell sensing system in serum. The curves were obtained with 2-hydroxy-3',4'-dichlorobiphenyl in 90% water/10% DMSO (■) or 90% serum/10% DMSO (▼). The bioluminescence signals have been corrected with respect to the blank. Values represent the means \pm standard deviation of triplicate determinations.

interacting with the walls of the plastic tube, thus making the analyte more available for diffusion into the cells. Overall, the resultant bioluminescence response of the whole-cell sensing system to the model compound in serum demonstrates the feasibility of direct detection of OH-PCBs in serum samples.

In real samples, PCB metabolites are present as mixtures. Various contamination patterns of hydroxylated PCBs have been reported in the literature for fish plasma [123], polar bear whole blood [124] and human whole blood [125], plasma [126-129], serum [114, 130] and cerebrospinal fluid samples [108]. Additionally, the ratios of total OH-PCBs to total PCBs calculated were around 4-56 % in human plasma [126] or even found to be 4-8 times higher in the whole blood of polar bears [124]. In our laboratory, the responses of the whole-cell sensing system to a model mixture of 10 OH-PCBs were tested. The obtained dose-response curve with a detection limit of 5.0×10^{-8} M suggests the potential for employing this sensing system as a screening method for multiple OH-PCBs contaminated samples. Notably, the application of this whole-cell sensing system to the detection of OH-PCBs in biological and environmental samples could remarkably reduce the analysis time and cost posed by currently used conventional methods, such as GC or LC methods. Further studies utilizing this whole-cell sensing system will enable us to monitor the bioavailable PCB metabolites in large pools of environmental and biological samples. This will provide a new insight into the pollution status at certain sites for better understanding of the current ecological conditions of the living environment.

CONCLUSIONS

In this work, a rapid and sensitive sensing system for the detection of hydroxylated PCBs based on genetically engineered whole cells has been developed. Additionally, the performance of this whole-cell sensing system has been demonstrated in a biological sample matrix. The results achieved suggest that the sensing system may find applications in biomedical analysis as well as in the environmental monitoring of hydroxylated PCBs. The ruggedness of the proposed sensing system can be further improved by employing lyophilized cells, which can be easily stored and transported to the field, and reconstituted for later use. Upon miniaturization and integration into an appropriate assay platform, we envision this system being developed into a rapid, high-throughput, field-portable method for the detection of hydroxylated PCBs in both environmental and biological samples.

ACKNOWLEDGMENTS

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CHAPTER THREE

ENGINEERING THE TRANSCRIPTIONAL REGULATORY PROTEIN, HbpR, TO DEVELOP A SCREENING METHOD FOR HYDROXYLATED POLYCHLORINATED BIPHENYLS

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a class of 209 congeners of persistent, toxic organochlorine compounds that have been exhaustively shown to contribute to a variety of negative health outcomes in humans. Specifically, PCBs have been shown to affect neurological and cognitive development,[131, 132] increase the risk of childhood leukemia,[133] reduce the success of *in vitro* fertilization attempts,[134] and cause endocrine system disruption.[135, 136] These compounds are nearly ubiquitous in the environment, as well as present in some homes and buildings due to their many years of use in applications such as plasticizers, surface coatings, inks, adhesives, flame retardants, duplicating paper, wire insulators, caulking materials, elastic sealants, heat insulation, and electrical transformers.[137] PCBs are classified as persistent organic compounds, meaning that once present in the environment they are not easily degraded by natural physical, chemical or biological processes. Additionally, PCBs are not readily eliminated from most complex organisms and exhibit a high degree of lipophilicity. As a result, there is a bioaccumulation effect for PCBs and an increase in exposure throughout the food chain (biomagnification) and

susceptibility of humans to exposure both from the environment and from dietary sources.

Upon human exposure to PCBs, hydroxylated metabolites are formed *in vivo* by cytochrome p450 enzymes.[138] The resulting hydroxylated PCBs (OH-PCBs) are less lipophilic, thus resulting in a decreased half-life in the body from an estimated median value of 37 years[139] to a half-life on the order of days.[140] Studies have shown that OH-PCBs may possess unique toxicity, acting as endocrine disruptors,[141] increasing the formation of reactive oxygen species,[142] and disrupting the membrane potential of neocortical cells.[143] While OH-PCBs as a class have a relatively short biological half-life, some congeners, particularly those derived from highly chlorinated parent PCBs, are not easily eliminated from the body.[144] The shortened half-life and increased hydrophilicity of OH-PCBs contribute to a lower total concentration in body fluids and tissues when compared to their parent PCBs. Typically, the total OH-PCB concentration is approximately an order of magnitude lower than the total PCB concentration when measured in serum.[140] Since the serum concentration of OH-PCBs corresponds to that of PCBs, OH-PCBs can serve as a biomarker for PCB exposure.

Previously, we have developed a whole-cell biosensing system for OH-PCBs employing the regulatory protein HbpR from *Pseudomonas azelaica*. [4] HbpR is a member of the XylR/DmpR subfamily of σ^{54} -dependent regulatory proteins (figure 3.1).[145] Acting as a negative genetic regulator, HbpR undergoes a conformational change, releases from the operator region, and

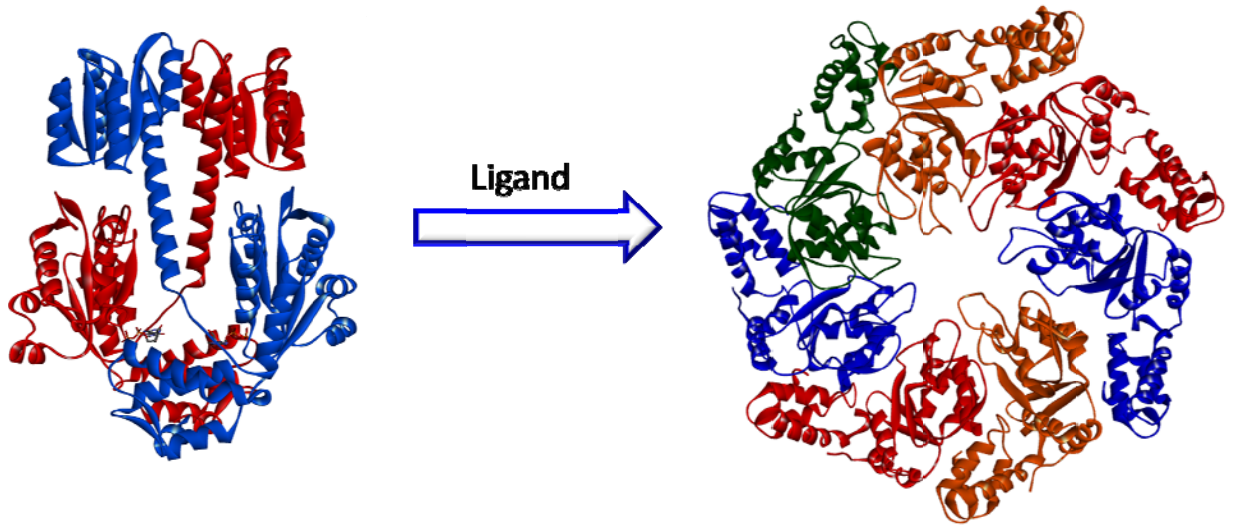


Figure 3.1. **Model of the conformational change for the protein, NtrC1.** The protein HbpR belongs to a family of proteins known as σ^{54} -dependent transcriptional regulators. The only member of this protein family with available structural information is NtrC1, shown above. These proteins consist of multiple domains: the effector-binding A-domain, the Q-linker B-domain, the ATPase active C-domain, and the DNA-binding D-domain. Upon effector binding, the proteins undergo a drastic conformational change originating in the A-domain, which results in the activation of C-domain ATPase activity, and release from the operator sequence by the D-domain.

allows transcription of genes under the control of the corresponding promoter upon effector binding. We hypothesize that we can exploit this conformational change in the development of a protein-based biosensing system for the detection of OH-PCBs. By covalently attaching an environmentally-sensitive fluorescent molecule to cysteines in HbpR, the fluorescent signal may change in a dose-dependent manner upon OH-PCB binding.

Protein-based sensing systems have the distinct advantage of typically producing a sample-to-answer response much faster than cell-based sensing systems. Previous work with HbpR suggests that the protein binds to most OH-PCBs, albeit with different affinities.[4] As a result of this class-specificity and the relatively fast assay times associated with protein-based sensing systems, we believe that a biosensing system based on HbpR is well-suited for the development of a screening method for large numbers of samples to select positive samples for more labor-intensive and time-consuming analysis by traditional methods, such as, gas chromatography/mass spectrometry. In this work, we describe the protein engineering and fluorescent labeling of HbpR, and the investigation of its response to OH-PCBs in buffer as well as in spiked human serum.

EXPERIMENTAL

Reagents. Custom oligonucleotide primers were purchased from Operon Biotechnologies (Huntsville, AL). BamHI, EcoRI, HindIII, T4 DNA ligase, and Pfu

DNA polymerase were purchased from Promega (Madison, WI). Luria-Bertani (LB) broth and agar, 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-HCl), sodium chloride, tris(2-carboxyethyl)phosphine and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Fair Lawn, NJ). OH-PCB reference standards for the compounds listed in table 3.1 were purchased from AccuStandard (New Haven, CT). Ampicillin sodium salt, tetracycline, and human serum were purchased from Sigma (St. Louis, MO). Urea, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and glycerol were purchased from BDH/VWR (Bridgeport, NJ). Dimethylsulfoxide (DMSO) was purchased from J.T. Baker (Phillipsburg, NJ). Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). Maltose was purchased from Acros (Pittsburg, PA). QIAquick gel purification kit and QIAprep DNA isolation kit were purchased from Qiagen (Valencia, CA). 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS), TOP10F' cells, and the vector pRSET_A were obtained from Invitrogen (Carlsbad, CA). Ten thousand molecular weight cut-off dialysis cassettes were purchased from Pierce (Rockford, IL). Amylose resin and the vector pMal-p4E were purchased from New England Biolabs (Ipswich, MA).

Apparatus. PCR reactions were carried out using an Eppendorf Mastercycler personal thermocycler (Hamburg, Germany). Optical density (OD) measurements were performed using a Milton Roy Spectronic 21D spectrophotometer (Ivyland, PA). Cell lysis was performed using a Fisher Sonic

Table 3.1. Assay response of selected OH-PCBs

Compound	Detection Limit (M)	Apparent K_D (M)	Dynamic Range (M)
2,3-Dihydroxy-3',4'-dichlorobiphenyl	N/A	N/A	N/A
2-Hydroxy-2',3',4',5,5'-pentachlorobiphenyl	1.0×10^{-6}	2.5×10^{-6}	$1.0 \times 10^{-6} - 1.0 \times 10^{-4}$
2-Hydroxy-2',3,4',5',6-pentachlorobiphenyl	1.0×10^{-6}	2.4×10^{-6}	$1.0 \times 10^{-6} - 1.0 \times 10^{-5}$
2-Hydroxy-2',3',4',5-tetrachlorobiphenyl	1.0×10^{-7}	2.1×10^{-6}	$1.0 \times 10^{-6} - 1.0 \times 10^{-5}$
2-Hydroxy-2',3',5',6'-tetrachlorobiphenyl	1.0×10^{-8}	2.4×10^{-7}	$1.0 \times 10^{-8} - 1.0 \times 10^{-6}$
2-Hydroxy-2',4',5,6'-tetrachlorobiphenyl	1.0×10^{-7}	1.3×10^{-7}	$1.0 \times 10^{-7} - 1.0 \times 10^{-6}$
2-Hydroxy-2',4',6-trichlorobiphenyl	3.2×10^{-7}	1.0×10^{-6}	$3.2 \times 10^{-7} - 1.0 \times 10^{-5}$
2-Hydroxy-3',4'-dichlorobiphenyl	1.0×10^{-7}	2.3×10^{-7}	$1.0 \times 10^{-7} - 1.0 \times 10^{-5}$
2-Hydroxy-3',5,5'-trichlorobiphenyl	N/A	N/A	N/A
4-Hydroxy-2',3,4',5-tetrachlorobiphenyl	1.0×10^{-6}	2.5×10^{-6}	$1.0 \times 10^{-6} - 1.0 \times 10^{-4}$
4-Hydroxy-2',3',5-trichlorobiphenyl	1.0×10^{-7}	1.0×10^{-6}	$1.0 \times 10^{-7} - 1.0 \times 10^{-5}$
4-Hydroxy-2',4',6'-trichlorobiphenyl	1.0×10^{-7}	9.6×10^{-7}	$1.0 \times 10^{-7} - 1.0 \times 10^{-5}$
4-Hydroxy-3,3',4',5-tetrachlorobiphenyl	1.0×10^{-7}	1.0×10^{-6}	$1.0 \times 10^{-7} - 1.0 \times 10^{-5}$
4-Hydroxy-3,3',5'-trichlorobiphenyl	1.0×10^{-8}	6.1×10^{-7}	$1.0 \times 10^{-8} - 1.0 \times 10^{-5}$
4-Hydroxy-3,5-dichlorobiphenyl	1.0×10^{-8}	5.6×10^{-8}	$1.0 \times 10^{-8} - 1.0 \times 10^{-6}$

Dismembrator 500 from Fisher (Fair Lawn, NJ). Centrifugation steps were carried out using a Beckman Coulter Avanti J-E centrifuge from Beckman Coulter (Palo Alto, CA). Fluorescence measurements were performed using a BMG Labtech POLARstar Optima microtiter plate fluorimeter from BMG (Offenburg, Germany).

Construction of pHbpR-A-MBP plasmid. For all cloning and culturing steps, cells were grown in LB supplemented for selection as described below. Cell cultures were grown at 37 °C, with shaking at 250 rpm. Initially, the gene for the A-domain of HbpR (*hbpR-A*) was amplified by PCR using the procedure provided by the supplier of the Pfu polymerase. The primers used were HbpR-A-BamHI (5'-GGTGGTGGATCCATGAAATCAAATAAAAATAATAGC-3') and HbpR-A-EcoRI (5'-GGTGGTGGATTCCGCCACATTTCCGGCGGGCTTCGC-3'). The template DNA used was pHYBP109 and was obtained from our previous work.[4] PCR reaction conditions were carried out as described by the supplier's protocol. Briefly, the reaction mixture contained final concentrations of 2.5 µM for each primer and 25 ng/µL of template DNA. The temperature program used for amplification consisted of an initial denaturation step of 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 65 °C for 2 min, and 72 °C for 2 min and a final elongation step of 72 °C for 5 min.

The resulting PCR product was purified by agarose gel electrophoresis, and the corresponding DNA band (617 bp) was excised from the gel and purified using the QIAquick Gel Extraction Kit. The purified PCR product, in tandem with the vector pRSET_A, was then digested with the restriction enzymes BamHI and

EcoRI following the supplier's protocol. The digested fragments were purified by agarose gel electrophoresis, co-purified using the QIAquick Gel Extraction Kit, and ligated using T4 ligase following the supplier's protocol. The resulting ligation product was transformed into TOP10F' cells, which were grown on LB agar with ampicillin (100 µg/mL) and tetracycline (15 µg/mL) for selection.

Transformation colonies were grown overnight in LB media with ampicillin and tetracycline. Plasmid from these cultures was isolated using the QIAprep kit, digested with BamHI and EcoRI, and analyzed by agarose gel electrophoresis for the correct plasmid, pRSET_A-hbpR-A. The purified plasmid possessing bands of the correct size was sequenced for confirmation. For transformants containing plasmids with the correct sequence, glycerol stocks were prepared and stored at -80 °C.

To prepare a plasmid for expression of the A-domain of HbpR (HbpR-A) as a fusion to maltose binding protein (MBP), the plasmids pRSET_A-hbpR-A and pMal-p4E were digested with BamHI and HindIII. The resulting products were purified by agarose gel electrophoresis, co-isolated from the gel using the QIAquick Gel Extraction Kit and ligated using T4 ligase as above. The resulting product was transformed into TOP10F' cells and grown on LB agar with ampicillin and tetracycline for selection. As above, the transformants were analyzed for the correct plasmid, pMal-p4E-hbpR-A, and candidates were sent for confirmation by DNA sequencing. Transformants containing the correct plasmid were preserved as glycerol stocks at -80 °C.

Expression and Purification of HbpR-A-MBP. For expression of HbpR-A-MBP, a 2.0 mL culture of TOP10F' cells containing pMal-p4E-hbpR-A was grown overnight in LB media supplemented with ampicillin (100 µg/mL) and tetracycline (15 µg/mL). Two 500 mL expression cultures in LB media were inoculated the following day using the overnight cultures and grown to an OD₆₀₀ of 0.4-0.5, and protein expression was induced by the addition of 1 mM IPTG. Expression was carried out overnight at 37 °C. Following expression, the cultures were centrifuged to a pellet (10,000 x g, 20 min, 4 °C), and the supernatant was discarded. The isolated cell pellet was used to prepare purified HbpR-A-MBP fusion protein.

The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and placed on ice. Cells were lysed on ice by pulsed sonication (10 s on, 10 s off, 10 min total), and the resulting suspension was immediately centrifuged (10,000 x g, 20 min, 4 °C) to separate the protein-containing supernatant from the cellular debris. The supernatant was added to a gravity-flow column containing 2 mL of amylose resin equilibrated according to the supplier's instructions, and the flow-through fraction was collected. The column was washed with 16 mL of lysis buffer, and the fraction was collected. Finally, the fusion protein was eluted from the column in 9 aliquots of 0.5 mL of lysis buffer containing an increasing concentration of maltose (1 mM for fraction 1, 2 mM for fraction 2, 5 mM for fraction 3, and 10 mM for fractions 4-9). All purification fractions were analyzed by SDS-PAGE electrophoresis to determine the purity of the expressed protein. Fractions containing significant impurities

were discarded, and purified protein was combined and dialyzed in 1 L of labeling buffer (20 mM HEPES, pH 7.2) overnight at 4 °C.

Labeling HbpR-A-MBP with IAEDANS. The thiol-reactive, environmentally-sensitive fluorophore IAEDANS was used for protein labeling. Protein modification was carried out according to the Invitrogen Molecular Probes handbook for thiol-reactive probes. Briefly, the protein was denatured and possible disulfide bonds were reduced by reacting 3.0 mL of the protein in labeling buffer containing 1 mM TCEP and 6 M urea for 2 h at 4 °C with stirring. Following this reaction, 10 µL of a 2.3×10^{-2} M stock of IAEDANS in DMSO was added to the same vial, resulting in a 10-fold molar excess of IAEDANS with respect to the number of cysteines present. This mixture was allowed to react in an amber glass vial in the dark overnight at 4 °C with stirring. Following the labeling reaction, the solution was dialyzed extensively at 4 °C to remove any free IAEDANS from solution and to gradually remove urea and allow protein refolding. The reaction solution was placed inside of a 10,000 molecular weight cut-off, 10 mL dialysis cassette and dialyzed in an initial dialysis buffer (20 mM HEPES, 2 M urea, 0.2 mM EDTA, pH 7.4) for 7 h at 4 °C with mixing in the dark. Following this, consecutive buffer changes were performed by removing half of the dialysis buffer and replacing with dialysis buffer with no urea, halving the previous urea concentration. Each dialysis step was carried out for at least 4 h. This was repeated 5 times, and then a final dialysis was carried out replacing the entire volume of dialysis buffer. The thoroughly dialyzed protein solution was

then removed from the dialysis cassette, placed in an amber glass vial, and stored at 4 °C.

Fluorescence Assays of OH-PCBs in Buffer. For fluorescence assays in buffer, stock solutions of selected OH-PCBs were prepared at a concentration of 1×10^{-2} M in DMSO. From these stock solutions, standards were prepared by serial dilution in DMSO resulting in a range of concentrations from 1×10^{-2} M to 1×10^{-8} M. Using these DMSO standards, assay standards were prepared by diluting each DMSO standard 1:10 into assay buffer (10 mM HEPES, pH 7.5), resulting in assay standards with a range of concentrations from 1×10^{-3} M to 1×10^{-9} M. An assay blank was also prepared by preparing a 10% (v/v) solution of DMSO in assay buffer. To perform assays of the compounds, 180 μ L of the HbpR-A-MBP-IAEDANS solution at a concentration of 1×10^{-7} M was mixed with 20 μ L of assay standards, as well as a blank sample, in triplicate. The fluorescence was measured using a BMG Labtech Polarstar Optima microtiter plate fluorimeter. Data were plotted using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) with a three-parameter sigmoidal curve fitting equation, and apparent binding constants were derived from this equation. Detection limits were determined as the concentration tested that produced a signal intensity that is at least 3 standard deviations above the blank signal.

Fluorescence Assay of 2-Hydroxy-3',4'-dichlorobiphenyl in Human Serum.

The assay of 2-hydroxy-3',4'-dichlorobiphenyl was carried out in human serum. The procedure was carried out as above for the preparation of standards, except that in the preparation of assay standards, purchased human serum was used

instead of buffer. The resulting serum samples contained the OH-PCB in a concentration range of 1×10^{-3} M – 1×10^{-9} M. Using these standards, the assay procedure above was followed precisely.

RESULTS AND DISCUSSION

HbpR is a member of the XylR/DmpR subclass of the NtrC family of regulatory proteins from bacteria.[146] This family of proteins typically contains four distinct domains (figure 3.1). The effector binding domain, or A-domain, interacts directly with an effector molecule and begins a drastic conformational change, which is relayed to the rest of the protein through a helix linker region in the B-domain. The conformational change is then translated to the C-domain, whose ATP-ase activity is triggered, which then recruits the σ^{54} RNA polymerase. Simultaneously, the DNA-binding D-domain undergoes a conformational change, which triggers its release from the operator region of the DNA resulting in expression of genes under the control of the associated promoter.[146] In the subclass of proteins encompassing XylR, DmpR, and HbpR, the effector molecule is aromatic, and it has been demonstrated that the isolated A-domain binds to its aromatic effector molecule *in vitro*.[147]

Members of this class of proteins are particularly difficult to express, purify, and stabilize in solution, and as a result structural information for members of this class of proteins has been difficult to acquire.[145, 148] Any structural information that has been obtained thus far is the result of modeling the structure of these proteins using as a template other proteins, which are very distantly

related and lack a high degree of homology.[145, 148] This lack of specific information on the overall protein structure, coupled with the lack of detailed information on the conformational change that occurs upon effector molecule binding makes it nearly impossible to rationally-design a protein that will generate a measurable change in signal upon analyte binding.

As an alternative, we have taken a shot-gun approach by attaching a fluorophore simultaneously to cysteines present in HbpR. Several strategies for expressing and purifying in significant quantities either the entire HbpR protein, or the isolated A-domain, HbpR-A were attempted. Initially, the genes for the expression of HbpR and HbpR-A were cloned into the expression vector pRSET_A. This expression system is designed to express the protein with an N-terminal histidine tag. This system yielded small amounts of insoluble protein for both HbpR and HbpR-A; subsequent attempts to denature and refold the protein were unsuccessful (data not shown). In order to improve protein solubility, we cloned the genes for both the full-length and truncated protein into the expression vector pMAL-p4E, which expresses the protein as a C-terminal fusion to MBP. Maltose binding protein possesses exceptional solubility and often confers this property upon its protein fusion partner. Indeed, when expressed as a fusion we were able to obtain soluble, purified HbpR-A-MBP. The HbpR-MBP fusion protein was insoluble upon expression. Attempts to cleave HbpR-A from MBP and purify isolated HbpR-A were also unsuccessful, as the protein immediately precipitated. For these reasons, this work focuses on the use of the HbpR-A-MBP fusion protein.

Extensive characterization of HbpR has been carried out previously. The selectivity of HbpR has been well-defined by initial work carried out by Jaspers *et al.* and expanded by our previous work.[4, 146] HbpR shows the greatest response to its native effector, 2-hydroxybiphenyl, but also has been shown to respond to some structurally related compounds such as 2,2'-dihydroxybiphenyl, 2-aminobiphenyl, 2-hydroxydiphenylmethane and many OH-PCBs. However, all previous work with this protein was accomplished in a cell-based sensing approach. The isolated protein has never been directly used *in vitro* to investigate the binding of any of these molecules.

The purified fusion protein was labeled with an environmentally-sensitive fluorophore, IAEDANS. An initial assay using this protein, HbpR-A-MBP-IAEDANS, with standards of the compound 2-hydroxy-3',4'-dichlorobiphenyl in buffer demonstrated that the protein did indeed respond in a dose-dependent manner (figure 3.2), with a limit of detection of 1.0×10^{-7} M. The assay for 2-hydroxy-3',4'-dichlorobiphenyl was also carried out in spiked human serum. A concentration-dependent response was observed in both sample matrices, although the blank-subtracted fluorescence intensity was slightly lower in serum samples, and the detection limit was an order of magnitude higher. This could be because the analyte may associate with proteins in serum making it less available to the protein-based sensor. However, the ability to detect OH-PCBs directly in serum with no sample pretreatment is a significant improvement over current detection methods.

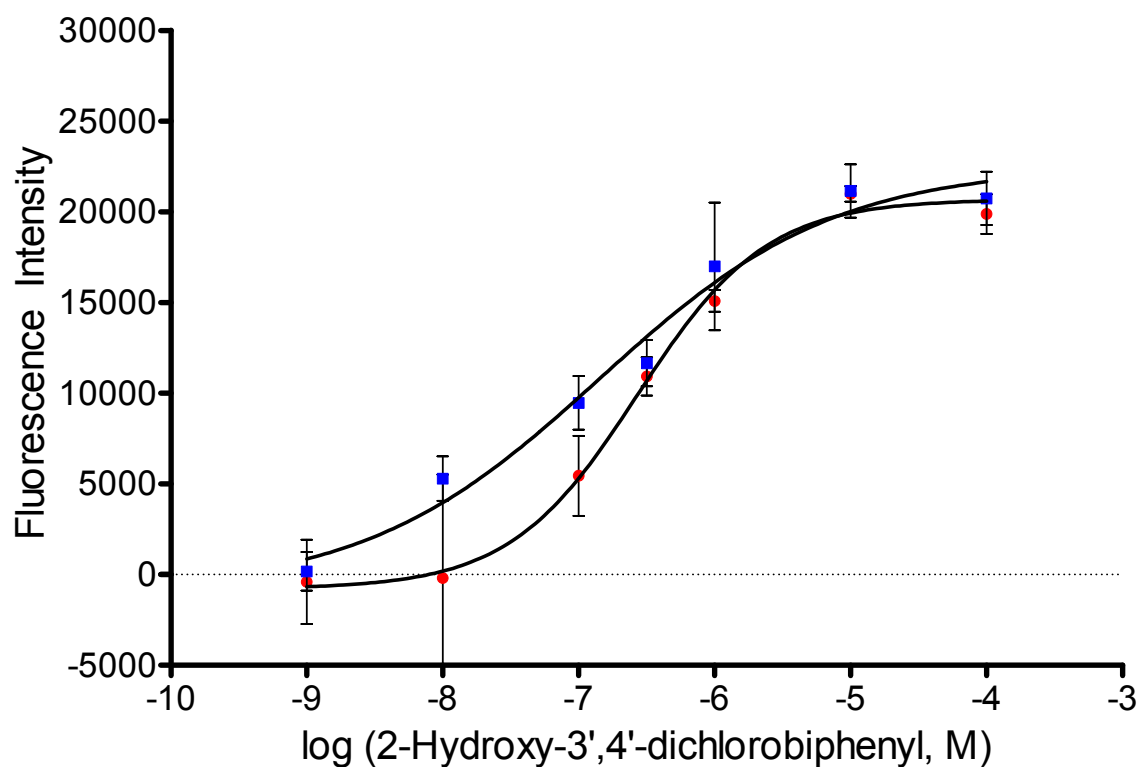


Figure 3.2. **Dose-response curves for 2-Hydroxy-3',4'-dichlorobiphenyl in buffer and spiked human serum.** The assay was carried out by mixing 20 μL of each standard in either buffer (■) or serum (●) with 180 μL of 1×10^{-7} M HbpR-A-MBP-IAEDANS and measuring immediately after mixing. The samples were prepared in triplicate, the results were averaged, and the signal of the blank was subtracted. The results were fitted with a sigmoidal response curve. Error bars denote +/- one standard deviation.

To investigate the effect of incubation time on assay performance, the signal from a single microtiter plate assay was measured immediately after addition of the analyte. Additional fluorescence measurements of the same microtiter plate were performed at 15 min, 45 min, and 105 min. While there was a gradual decrease in the total fluorescence intensity of the measurements with time, the detection limit and dynamic range were not improved at longer incubation times (Figure 3.3). For this reason, all measurements were performed with no additional incubation time.

In order to investigate the response characteristics of the biosensing system, additional assays were carried out for the detection of other OH-PCBs as listed in table 3.1. The biosensing system responded to most of the OH-PCBs tested, with the exception of 2-hydroxy-3',5,5'-trichlorobiphenyl and 2,3-dihydroxy-3',4'-dichlorobiphenyl. In previous work in our laboratory, our whole-cell sensing system seemed to respond to the former. It is unclear why the isolated protein would fail to respond to this compound. However, our whole-cell sensing system failed to respond to any dihydroxy compounds, so it is unsurprising that the isolated protein shows no response to the latter. Since in our protein biosensing system, HbpR-A is expressed as a fusion to MBP, which contains one cysteine that could be modified with IAEDANS, we conducted the assay using MBP-IAEDANS with 2-hydroxybiphenyl to confirm that no response was observed from MBP alone (data not shown). This was done to ensure that the response we were observing was due to HbpR-A and not to MBP.

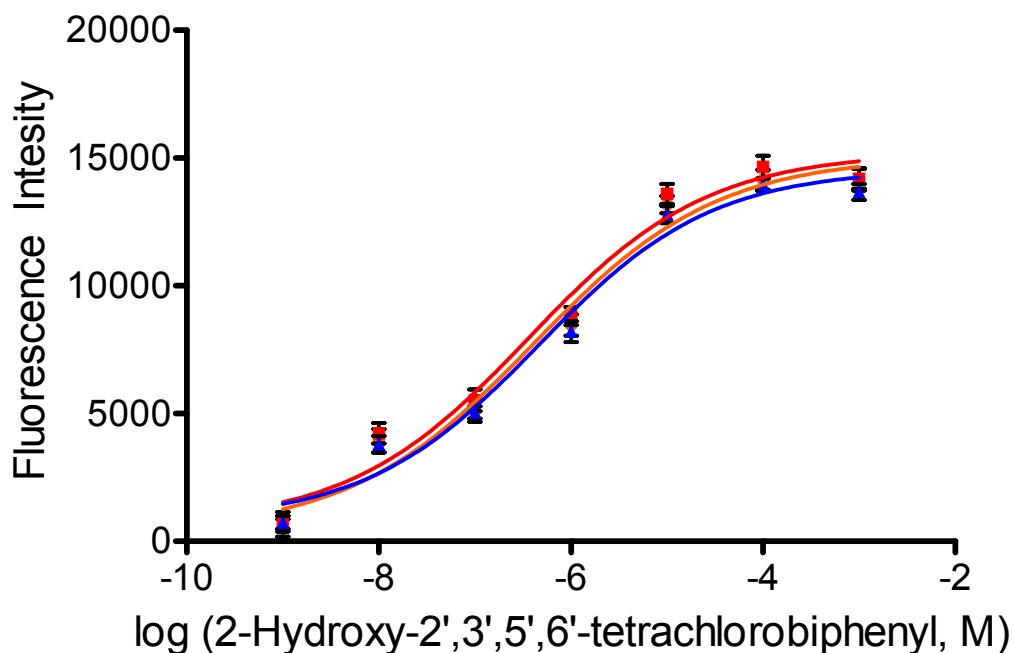


Figure 3.3. **Time study of 2-Hydroxy-2',3',5',6'-tetrachlorobiphenyl in buffer.**

The assay was carried out by mixing 20 μL of each standard in buffer with 180 μL of 1×10^{-7} M HbpR-A-MBP-IAEDANS and measuring immediately (●), after 45 min (■), and after 105 min (▲). The samples were prepared in triplicate, the results were averaged, and the signal of the blank was subtracted. Error bars denote +/- one standard deviation.

Detection limits for the compounds assayed ranged from 1×10^{-8} M to 1×10^{-6} M, and the dynamic range varied from one to three orders of magnitude (Table 3.1). The percent relative standard deviation (%RSD) was calculated for each of the data points for all of the compounds as an evaluation of intra-assay reproducibility. The %RSD ranged from 0.01 to 10.8. Additionally, the %RSD was calculated using data from repeated assays of the same compound, 2-hydroxy-3',4'-dichlorobiphenyl in order to evaluate inter-assay variability and found to range from 1.1 to 2.3. These data suggest that the assay is reproducible.

It is hypothesized that the differing response to various OH-PCBs is due to structural differences between the molecules, which would affect their interaction with the binding pocket of HbpR-A. Without the availability of detailed structural information of the protein only inferences can be made to explain the variations in response. To gain some insight into the observed variation in the response to different compounds, correlation analysis was performed on the data. Spearman correlation analyses were performed on the basis of a number of characteristics that differentiate OH-PCB congeners (Table 3.2). First, the dihedral angle between the two aromatic rings of each molecule was calculated. This was accomplished by modeling the energy-minimized 3D structure of the compounds using Chem3D Pro 12.0 (CambridgeSoft, Cambridge, MA). The dihedral angle reflects the planarity of each molecule. In general, substitutions in the ortho-positions on each ring contribute to a less planar conformation for the OH-PCBs.

Table 3.2. **Molecular characteristics of selected OH-PCBs**

Compound	Volume (Å ³)	logP	Dihedral Angle (°)
2,3-Dihydroxy-3',4'-dichlorobiphenyl	171.3	4.25	22
2-Hydroxy-2',3',4',5,5'-pentachlorobiphenyl	209.3	6.62	42
2-Hydroxy-2',3,4',5',6-pentachlorobiphenyl	210.6	6.62	56
2-Hydroxy-2',3',4',5-tetrachlorobiphenyl	195.0	6.01	41
2-Hydroxy-2',3',5',6'-tetrachlorobiphenyl	197.7	5.59	60
2-Hydroxy-2',4',5,6'-tetrachlorobiphenyl	197.6	5.64	57
2-Hydroxy-2',4',6-trichlorobiphenyl	182.5	5.38	54
2-Hydroxy-3',4'-dichlorobiphenyl	164.9	4.75	21
2-Hydroxy-3',5,5'-trichlorobiphenyl	179.1	5.40	22
4-Hydroxy-2',3,4',5-tetrachlorobiphenyl	193.4	6.01	29
4-Hydroxy-2',3',5-trichlorobiphenyl	179.2	5.38	31
4-Hydroxy-2',4',6'-trichlorobiphenyl	180.6	4.78	44
4-Hydroxy-3,3',4',5-tetrachlorobiphenyl	192.2	6.01	1
4-Hydroxy-3,3',5'-trichlorobiphenyl	178.6	5.40	0
4-Hydroxy-3,5-dichlorobiphenyl	163.6	4.73	0

The solvent exclusion volume that each molecule would be expected to occupy was also calculated using Chem3D Pro 12.0. This value may influence the affinity to the binding pocket, as molecules that are either too large or too small would not be expected to bind well. Lastly, the partition coefficient, logP, which is a reflection of the hydrophobicity of each molecule, was calculated using ChemDraw Ultra 12.0 (CambridgeSoft, Cambridge, MA). These values are predicted using a fragmentation method based on atomic contributions from a large reference set of molecules by least squares analysis. Since the effector molecules that bind to HbpR are hydrophobic, it is expected that the binding pocket of HbpR is a hydrophobic binding pocket located on the interior of the A-domain. Additionally, binding pockets located in the interior of proteins are typically a hydrophobic environment. For this reason, the hydrophobicity of different congeners may affect the binding interaction.

Correlation analyses for each of these parameters were carried out with respect to the detection limits and apparent K_D values observed in the assay data. There was no significant correlation calculated between either the detection limits or apparent K_D values and the dihedral angles or the molecular volumes. However there was a slight correlation ($r = 0.5885$, $p = 0.0344$) between the detection limits and the partition coefficients and an even stronger correlation ($r = 0.7406$, $p = 0.0038$) between the apparent K_D values and the partition coefficients (figure 3.4). This would suggest that as the hydrophobicity of the molecules increases so do both the detection limit and the apparent K_D value. A possible explanation of this behavior is that the position of the hydroxy

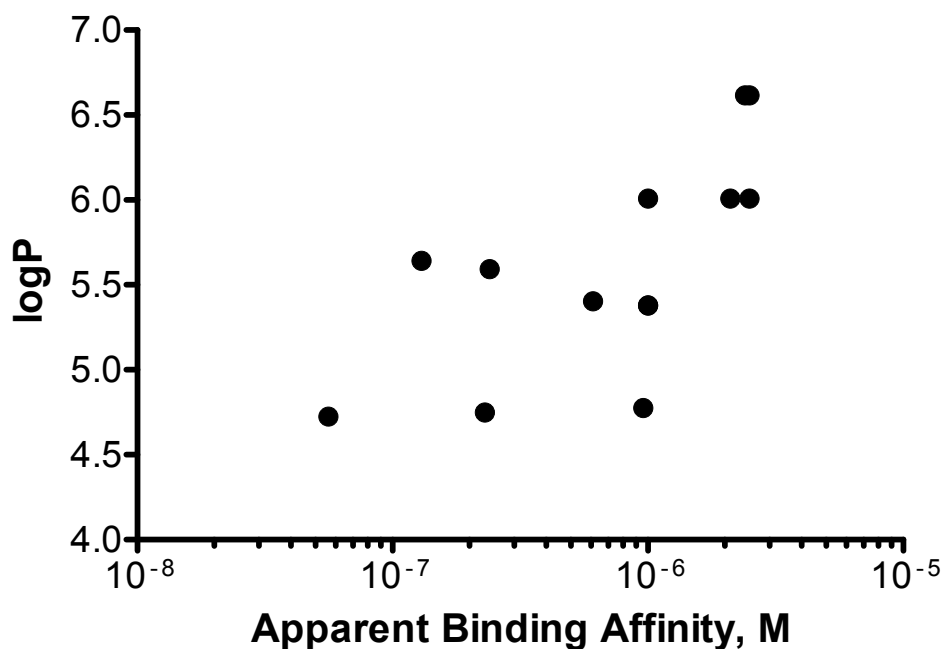


Figure 3.4. **Correlation analysis of logP values and apparent binding affinities.** Apparent binding affinities, K_D , were calculated by GraphPad Prism 5.0 software from sigmoidal response curves obtained from the assay of each compound. Values of logP were obtained using ChemDraw Ultra 12.0. A Spearman correlation analysis was performed between the two parameters using GraphPad Prism 5.0 software. The calculated correlation value was 0.7406 ($p = 0.0038$).

functionality is important and determines to some extent how HbpR interacts with the effector molecules. There may be one or more hydrophilic amino acids present within the binding pocket of HbpR that participate in hydrogen-bonding interactions with the hydroxy-group. Interestingly it appears that it is not the position of the chlorine substituents from various OH-PCBs that determines strength of interaction through steric hindrance, but rather the relative hydrophobicity of the OH-PCBs. This suggests that the binding pocket may be large and hydrophobic and as such be able to accommodate a wide range of structurally related compounds. To be able to ascertain this with a high degree of confidence would require the availability of the three-dimensional structure of HbpR.

CONCLUSION

We have developed a protein-based sensing system for the detection of OH-PCBs by the covalent attachment of the fluorophore IAEDANS to cysteines present in the effector binding domain of the transcriptional regulatory protein HbpR. We have demonstrated the dose-dependent response in buffer as well as in a sample matrix of human serum. This biosensing system responds broadly to most OH-PCBs tested resulting in detection limits as low as 1×10^{-8} M, which should enable its application as a rapid, cost-effective, high-throughput screening method for large numbers of samples suspected of containing OH-PCBs. This should lead to the identification of positive samples to be subjected to a more in-depth analysis by other analytical methods such as GC-MS. Ongoing structural

studies of HbpR should eventually provide more detailed structural information, which will provide for the rational design of the protein to improve its response characteristics and allow for the development of a biosensing system with more desirable properties, such as, improved detection limits, selectivity, and response to more highly chlorinated OH-PCBs.

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CHAPTER FOUR

ENGINEERING GLUCOSE RECOGNITION PEPTIDES FROM NATURE: SELECTIVE AND SENSITIVE BIOSENSORS FOR GLUCOSE

INTRODUCTION

The development of reliable, low cost technologies for glucose sensing has been an active field of research since the first glucose biosensing device was proposed in 1962.[96, 149] While recent generations of these devices have seen improvements in performance, the basic principles of the technology have remained almost identical. Current-generation commercially available glucose meters measure glucose by electrochemical methods based on the enzymes glucose oxidase (GOx) or glucose-1-dehydrogenase (GDH).[149] These electrochemical methods suffer from some significant drawbacks including hematocrit dependence, interference from other electrochemically active molecules, hypoxemia, or hypotension.[149] Lack of electrochemical selectivity is especially troublesome, as the list of potentially interfering compounds includes molecules commonly found in blood such as acetaminophen, salicylic acid, ibuprofen, ascorbic acid, etc.[96, 150]

To overcome the disadvantages of electrochemical methods for glucose sensing, methods based on alternative technologies have been proposed and are currently being evaluated. To that end, the design and development of reagentless optical sensing strategies that are sensitive, selective, reproducible,

accurate, rugged, and capable of glucose detection in the physiological concentrations and conditions is an active field of research. Among these methods are fluorescence-based,[89, 151, 152] fluorescence resonance energy transfer (FRET)-based,[151, 152] and bioluminescence-based[72] methods. In each of these cases, the glucose/galactose binding protein (GBP) (Figure 4.1) from *Escherichia coli* is employed as the biological recognition element. GBP is a well-studied periplasmic binding protein consisting of 309 amino acids, which undergoes a hinge-motion conformational change upon binding glucose or galactose. As outlined in the examples mentioned above, this conformational change has been exploited in a variety of ways to produce a measurable optical signal in response to glucose. As a result of these methods relying on an optical response, many of the drawbacks of electrochemically-based detection of glucose, such as interference from other molecules are circumvented.

While biosensing systems based upon GBP address some of the shortcomings of electrochemical-based systems, the relatively low dissociation constant (K_D) of GBP (in the micromolar range) compared to the physiological concentration of glucose (in the millimolar range) constrains GBP's widespread implementation into commercial devices. Efforts to alter the binding affinity of proteins by affecting changes in the amino acid sequence have included site-directed mutagenesis,[151, 153] random mutagenesis,[154] and DNA shuffling.[155] These efforts have resulted in proteins with affinities shifted to the millimolar range, however the selectivity toward glucose against other sugars and glycosylated moieties has not been fully determined. These approaches may

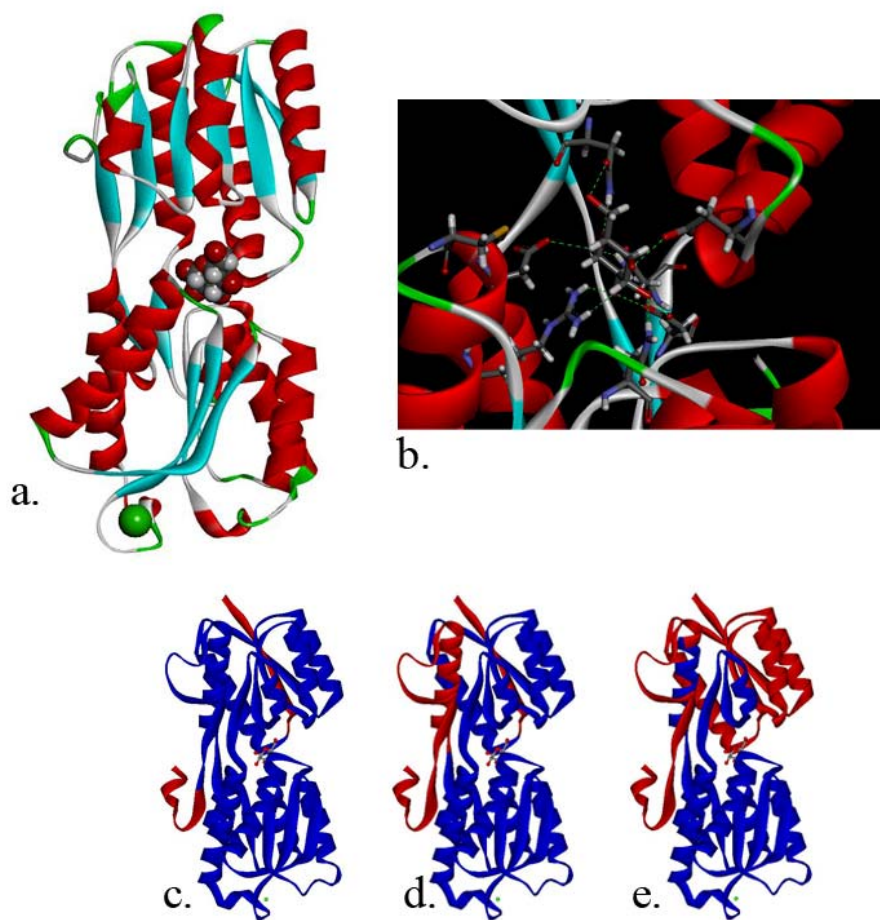


Figure 4.1. Protein structures for native GBP and tGRPs. Native GBP (a.) is shown with galactose in the ligand-binding pocket and calcium (shown in green) present in the calcium-binding pocket (PDB ID: 1GLG). In (b.), the binding pocket is magnified showing the amino acids involved in hydrogen bonding with glucose as well as Cys152. tGRP1 (b.), tGRP2 (c.), and tGRP3 (d.) are shown with truncated areas of the protein in red.

significantly alter the inherent selectivity of the protein, as they involve introducing significant changes into the binding pocket; thus the applicability of these systems for glucose sensing may be somewhat compromised.

In this work, we hypothesize that by rationally engineering the protein to truncate portions of GBP, we can construct truncated glucose recognition peptides (tGRPs), which maintain the wild-type GBP's response to glucose while altering the dissociation constant of the protein by affecting the overall folding and structure of the protein. In the design of these proteins, it is important to maintain much of the hydrogen-bonding network that is responsible for the direct interaction with glucose, as well as the hinge region connecting the two lobes, so that the integrity of the binding is preserved. Herein, we present a series of newly designed tGRPs, their characterization, and their use in glucose sensing.

EXPERIMENTAL PROCEDURES

Reagents. All oligonucleotide primers were purchased from Operon Biotechnologies (Huntsville, AL). Phusion DNA polymerase was purchased from New England Biolabs (Ipswich, MA). 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris-base), β -mercaptoethanol, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), calcium chloride (CaCl_2), ampicillin sodium salt, tetracycline, ethidium bromide, agarose, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and D-glucose, sucrose, and lactose, and D-galactose were obtained from Sigma-Aldrich (St. Louis, MO). Glycine, sodium chloride, ethylenediaminetetraacetic acid (EDTA), and Luria-Bertani (LB) broth and agar,

were purchased from Fisher Scientific (Fair Lawn, NJ). Methanol, acetic acid, sodium phosphate dibasic, sodium phosphate monobasic, glycerol, and bromophenol blue were purchased from VWR (Bridgeport, NJ). Imidazole and maltose were purchased from J.T. Baker (Phillipsburg, NJ). Isopropyl β -D-1-thiogalactopyranoside (IPTG) and dithiothreitol (DTT) were purchased from Gold Biotechnology (St. Louis, MO). Sodium dodecyl sulfate was ordered from Curtin Matheson (Houston, TX). The Bradford protein assay kit was purchased from Biorad (Hercules, CA). Ni-NTA agarose resin, QIAquick gel purification kit, QIAprep DNA isolation kit, and the pQE70 vector were purchased from Qiagen (Valencia, CA). TOP10F' cells, Tris-Glycine SDS PAGE gels, 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC), Mark12 protein standard, and 1 kb DNA standard were purchased from Invitrogen (Carlsbad, CA). Three thousand five hundred MWCO Slide-A-Lyzer 3-12 mL dialysis cassettes were purchased from Pierce (Rockford, IL). T4 DNA ligase, SphI restriction enzyme and BglII restriction enzyme were purchased from Promega (Madison, WI).

Apparatus. Polymerase chain reactions (PCR) were performed using an Eppendorf Mastercycler Personal Thermocycler (Hauppauge, NY). Electrophoresis of DNA was carried out using an FB105 Fischer Biotech Electrophoresis Power Supply (Pittsburg, PA). DNA gels were visualized using a UV Transilluminator platform from UVP (Upland, CA). Optical density measurements were obtained using a Spectronic 21D from Milton Roy (Ivy Land, PA). Cells were lysed using a 550 Sonic Dismembrator from Fisher Scientific

(Pittsburg, PA). Proteins were expressed by incubating bacteria at 37 °C on a Forma Scientific Orbital Shaker (Fairlawn, NJ). All centrifugations were carried out using a Beckman J2MI centrifuge (Palo Alto, CA). Proteins were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Invitrogen 10-20% Tris-glycine gels in an Invitrogen X Cell Sure Lock Mini Cell (Carlsbad, CA). Fluorescence measurements were obtained using a QuantaMaster 40 Spectrofluorometer from PTI (Birmingham, NJ). Circular dichroism measurements were taken using a Jasco J-810 Spectropolarimeter (Easton, MD). DNA sequencing was performed by the Advanced Genetic Testing Center at the University of Kentucky.

Cloning of Truncated Glucose Recognition Peptides (tGRPs). To prepare tGRPs, various segments of the full-length glucose binding protein (GBP) containing a mutation introducing a unique cysteine in place of histidine at position 152 from *Escherichia coli* (*E. coli*) were amplified by PCR and ligated into the expression vector pQE70. Specifically, gene fragments were amplified corresponding to amino acids 14-296 (tGRP1), 14-256 (tGRP2), and 87-271 (tGRP3) of the native sequence. For the amplification of tGRP1, primers tGBP(14-)fwd [5'-GGTGGTGCATGCGCGATAACTTTATGTCTGTAGTGCGG-3'] and tGBP(-296)rev [5'-GGTGGTAGATCTAACATAAGGTACGCGGACCAC-3'] were used. For the amplification of tGRP2, primers tGBP(14-)fwd and tGBP(-256)rev [5'-GGTGGTAGATCTGTTTCAGTACGGTGCCCGCCAG-3'] were used. For the amplification of tGRP3, primers tGBP(87-)fwd [5'-GGTGGTGCATGCGCGTGGTTTTCTTCAACAAAGAACCG-3'] and tGBP(-

271)rev [5'-GGTGGTAGATCTGTTTTTCGCCAGATCAAAGGTCGC-3'] were used. PCR was carried out using Phusion High-Fidelity DNA Polymerase. PCR conditions consisted of an initial denaturation period of 30 s at 98 °C. Next, 30 cycles of 98 °C for 30 s, 70 °C for 30 s, and 72 °C for 60 s followed by a final elongation period of 72 °C for 5 min were carried out. The resulting reaction products were analyzed by tris-acetate-EDTA (TAE) 1% agarose gel electrophoresis, and the appropriate DNA fragments (861 bp for tGRP1, 741 bp for tGRP2, and 567 bp for tGRP3) were excised from the gel and purified using the QIAquick gel extraction kit.

The DNA fragments tGRP1, tGRP2, and tGRP3 along with expression vector pQE70 were digested with restriction enzymes BglII and SphI. The resulting products were analyzed by TAE 1% agarose gel electrophoresis, excised from the gel, and purified using the QIAquick gel extraction kit. A gel slice of digested pQE70 was co-purified in each separate tube containing tGRP1, tGRP2, and tGRP3 allowing the vector and insert to be eluted from the purification column simultaneously. To the eluted DNA, T4 DNA ligase and T4 DNA ligase buffer (10x) were added, and the ligation reaction was allowed to proceed overnight at room temperature. The ligated DNA was transformed into TOP10F' cells. Plasmid DNA was isolated from overnight cultures of selected transformants and analyzed for the presence of the desired DNA fragments by digestion with BglII and SphI. DNA sequencing was also performed to confirm the correct DNA sequence.

tGRPs Expression and Purification. Plasmids tGRP1 and tGRP2 were transformed into TOP10F' chemically-competent cells. For protein expression, cells were grown overnight at 37 °C with shaking in 3 mL of LB broth containing ampicillin (100 µg/mL) and tetracycline (12.5 µg/mL) for selection. The following day in a 1 L flask, 500 mL of LB broth containing ampicillin (100 µg/mL) and tetracycline (12.5 µg/mL) was inoculated with the overnight culture and grown at 37 °C with shaking at 250 rpm to an OD₆₀₀ of 0.4-0.5. Protein expression was then induced with IPTG at a final concentration of 1.0 mM. The expression culture was grown overnight at room temperature with shaking.

For protein purification, the culture expressing the desired proteins was centrifuged to a pellet at 12000 x g for 20 min at 4 °C, and the supernatant was removed and discarded. The bacterial cell pellet was resuspended in 15 mL lysis buffer (50 mM NaH₂PO₄, 30 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication on ice using a programmed cycle of 10 s on, 10 s off, for 10 min total. The cell debris was pelleted by centrifugation 12000 x g for 20 min at 4 °C, and the resulting crude protein-containing cell-extract was removed to a separate culture tube. To the crude extract, 1.0 mL of Ni-NTA resin was added and mixed at 4 °C for 1 h. The solution was then added to a gravity-flow column, and the flow through was collected. The resin was washed with 20 mL of wash buffer (50 mM NaH₂PO₄, 30 mM NaCl, 20 mM imidazole, pH 8.0), and the wash fraction was collected. Purified protein was eluted from the column in 1.0 mL aliquots of elution buffer (50 mM NaH₂PO₄, 30 mM NaCl, 250 mM imidazole, pH 8.0). All

collected fractions were analyzed by SDS-PAGE electrophoresis, and fractions containing purified protein were combined and stored at 4°C.

tGRPs Modification with Fluorophore. Purified proteins were reacted with an excess of DTT to reduce possible disulfide bonds between two molecules of tGRP. Excess DTT was removed by dialysis in 3500 MWCO dialysis cassettes in dialysis buffer (10 mM HEPES, 0.2 mM CaCl₂, pH 8.0). The dialyzed protein was reacted with a 10-fold molar excess of MDCC dissolved in DMSO, using manufacturer's instructions. The labeling reaction was carried out overnight at 4 °C in an amber glass vial, protected from light. Following the labeling reaction, the protein was extensively dialyzed against dialysis buffer (as above) to remove any excess MDCC. Labeled proteins were stored at 4 °C, protected from light.

Assay of Glucose with MDCC-labeled tGRPs. For the glucose assay, MDCC-labeled protein was used at a final concentration of 1×10^{-7} M in assay buffer (10 mM HEPES, 0.2 mM CaCl₂, pH 8.0). Glucose standards were freshly prepared in assay buffer by serial dilution using a freshly prepared 0.1 M glucose solution. For the assay, 180 µL of the labeled protein solution was added to 20 µL of each standard, as well as a blank, in triplicate and mixed gently and thoroughly. Fluorescence was measured immediately in a 200 µL quartz microcuvette. MDCC was excited at a wavelength of 419 nm, and fluorescence emission collected at 466 nm. Instrument slit widths were set to 4 nm, step size was 0.5 nm, integration time was 0.1 s. Fluorescence at 466 nm was graphed versus glucose concentration and analyzed using GraphPad Prism 5.0 software.

Measurement of Far- and Near-UV Circular Dichroism Spectra. Far-UV and near-UV circular dichroism spectra were collected for tGRP1 and tGRP2 in both the presence and absence of glucose. Briefly, protein concentration was determined according to the method by Greenfield *et al.*[156]. Proteins were dialyzed in CD buffer (10 mM phosphate, 0.2 mM CaCl₂, pH 7.5). Protein samples were prepared at a concentration of 0.2 mg/mL with and without the presence of 100 mM glucose in CD buffer for far-UV experiments and 1.0 mg/mL for near-UV experiments. Blank solutions consisting of CD buffer were prepared with and without 100 mM glucose. Spectra were collected using a Jasco J-810 spectropolarimeter.

For far-UV experiments, spectra were collected at room temperature from 200-260 nm, the data pitch was set to 0.5 nm, scanning mode was set to continuous, scan speed was 20 nm/s, response was set to 8 s, bandwidth was 1 nm, and accumulations was set to 3. A quartz CD cuvette with a pathlength of 0.1 cm was used. Near-UV experiments were carried out at room temperature in a quartz cuvette with a 1.0 cm pathlength. Scans were performed with the settings mentioned above, however the wavelength range was 250-350 nm. Data for corresponding blanks were subtracted from each sample, and the results were converted to molar ellipticity. Spectra were created by plotting molar ellipticity versus wavelength using GraphPad Prism 5.0 software.

Determination of Protein Thermal Stability. The melting temperature of each of the tGRPs was determined by monitoring circular dichroism at a wavelength of 222 nm. Samples were prepared as described for the far-UV experiments.

Settings were the same as above. The temperature range was set from 10-90 °C at a slope of 2 °C/min. Data were plotted versus temperature using GraphPad Prism 5.0 software and normalized with respect to the signal at 70 °C.

RESULTS AND DISCUSSION

The glucose binding protein has been studied extensively as a possible sensing component of future generation of devices for the continuous, real-time monitoring of glucose for the management of diabetes.[72, 89, 151, 154] However, it has been noted that the native form of the protein has a binding affinity in the micromolar range, which is too low to be useful at physiologically-relevant glucose concentrations, which range from 2-20 millimolar. By truncating the structure of GBP, we hypothesize that the resulting perturbation in the structure and folding of the protein will result in an altered affinity for glucose. Truncation, like random and site-directed mutagenesis, may alter the stability, folding, activity, and binding interactions of a protein. It has been shown that truncated proteins can exhibit altered activity and binding affinities.[157, 158]

Previous work has determined the binding constant of native GBP for glucose to be 0.2 μM . [89] To investigate the effect of truncating GBP on the binding affinity and stability of the protein, three truncated versions of GBP were engineered. These three proteins, tGRP1, tGRP2, and tGRP3 consist of amino acids 14-296, 14-256, and 87-271, respectively, of native GBP (Figure 4.1). In order to maintain activity toward glucose, selectivity against other sugar

molecules, and the ability to undergo the correct conformational change, the native structure was truncated in such a way that most of the amino acids involved in hydrogen-bonding interactions within the binding site were maintained. Native GBP interacts with glucose through a network of hydrogen-bonding interactions involving Asp14, Asn91, His152, Asp154, Arg158, Asn211, Asp236, and Asn256 (Figure 4.1).[159] tGRP1 and tGRP2 were engineered to include all amino acids listed above. tGRP1 included all amino acids through residue 296 in order to maintain all three strands of the hinge region of the protein structure. In the design of tGRP2, all amino acids located after residue 256 were removed, thus maintaining all hydrogen-bonding amino acids as well as two of the three strands composing the hinge region. In the design of tGRP3, much of one lobe of the protein, one strand of the hinge region, and one amino acid from the binding pocket were removed. The three different proteins were expressed in *E. coli* and chemically modified via site-selective labeling of a unique Cys residue with a fluorescent coumarin probe, namely MDCC.

The truncated proteins were characterized in terms of their binding ability to glucose and to other sugars. Upon glucose binding, the fluorescence emission from the MDCC-labeled tGRPs was quenched. We hypothesize that in the absence of glucose (when the protein structure is more open) the fluorescent probe MDCC assumes a position within the glucose binding pocket. However, when glucose binds, MDCC is displaced from this position causing a change in fluorescence. Native GBP exhibits a K_D of 0.2 μ M. It was found that as the protein was increasingly truncated, the apparent K_D increased, indicating a

decreasing affinity for glucose (Figure 4.1) to the point that tGRP3 showed no response to glucose (data not shown). In the structure of native GBP, eight amino acids are directly involved in hydrogen-bonding as a first-shell type of interaction with glucose, and, in addition, ten more amino acids interact with the first-shell amino acids to stabilize their structure around the sugar ligand.[159] While both tGRP1 and tGRP2 maintain all of the first-shell interacting amino acids, part of their second-shell amino acids was removed. Specifically, in tGRP1 and tGRP2 one and four second-shell amino acids were removed respectively. As more of these stabilizing, second-shell amino acids are removed, it is likely that the hydrogen-bonding interactions with glucose become disrupted, thus resulting in an increased K_D of 75 μM for tGRP1 and 0.25 mM for tGRP2 (Figure 4.2).

Disrupting the first- and second-shell amino acids may also affect the selectivity of the proteins, causing them to respond to sugar molecules other than glucose and galactose. As the overall structure becomes less stable, it is

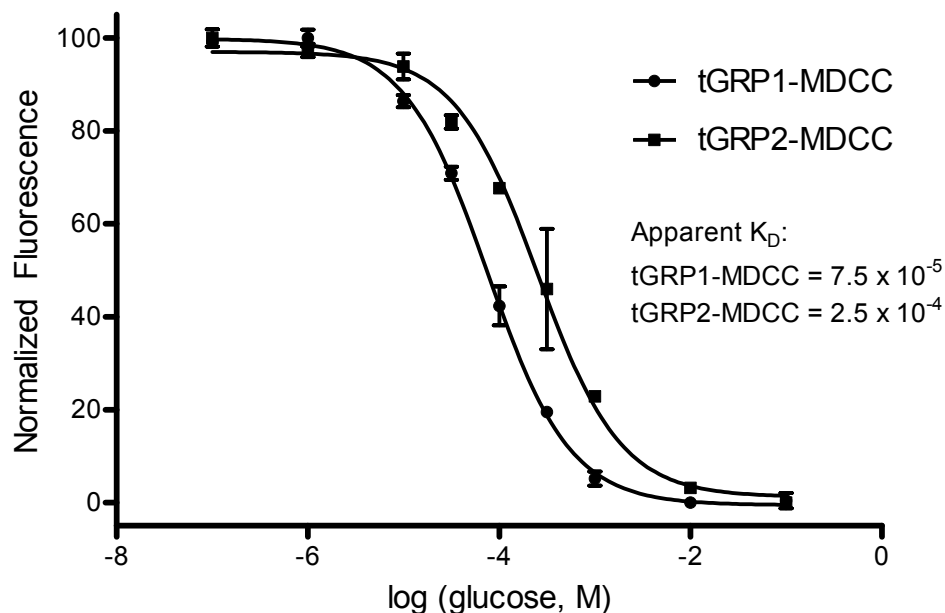


Figure 4.2. Normalized fluorescence response curve for tGRP1 and tGRP2.

The glucose-response curve for tGRP1 and tGRP2 labeled at position 152 (with respect to native GBP) with MDCC. MDCC-labeled protein was used at a final concentration of 1×10^{-7} M in buffer (10 mM HEPES, 0.2 mM CaCl_2 , pH 8.0). Glucose standards were prepared in buffer by serial dilution from a fresh 0.1 M glucose solution. For the assay, 180 μL of the labeled protein solution was added to 20 μL of each standard, as well as a blank, in triplicate and mixed gently and thoroughly and fluorescence was measured immediately. Data points represent the average of blank-subtracted triplicate samples. The apparent K_D for tGRP1 is 7.5×10^{-5} M and for tGRP2 is 2.5×10^{-4} M. The dynamic range for tGRP1 and tGRP2 is 1×10^{-3} M – 1×10^{-6} M and 1×10^{-2} M – 1×10^{-5} M respectively. Error bars denote +/- one standard deviation.

possible the binding pocket may become more flexible and able to accommodate other similarly-shaped ligands. To investigate this, a selectivity study with MDCC-labeled tGRPs was carried out with a variety of physiologically-relevant sugar molecules (Figure 4.3). As with native GBP, tGRPs responded best to glucose and to a lesser degree to galactose. None of the other sugar molecules showed a significant response.

To investigate whether the overall structural stability of the tGRPs was altered, the thermal stability of each tGRP was determined by circular dichroism (CD) spectroscopy. Protein thermal stability is an important consideration when developing protein-based sensors that will be used for extended periods of time at human body temperatures. Improved thermal stability will increase the lifetime of the sensor allowing for long-term, reproducible glucose determination. Native GBP has a melting temperature (T_m) of 66.9 °C in the absence of glucose and 70.2 °C in the presence of glucose.[160] Truncating the native structure had a significant effect on the thermal stability (Figure 4.4). In the absence and presence of glucose, tGRP1 was found to have a T_m of 39.7 °C and 44.2 °C respectively. The corresponding T_m values for tGRP2 were 37.3 °C and 43.2 °C. Since tGRP3 showed no glucose response, it was not characterized further. This drastic loss in thermal stability indicates significant structural changes in the protein structure that result from truncation, which is manifested as an increase in dissociation constants for the ligand.

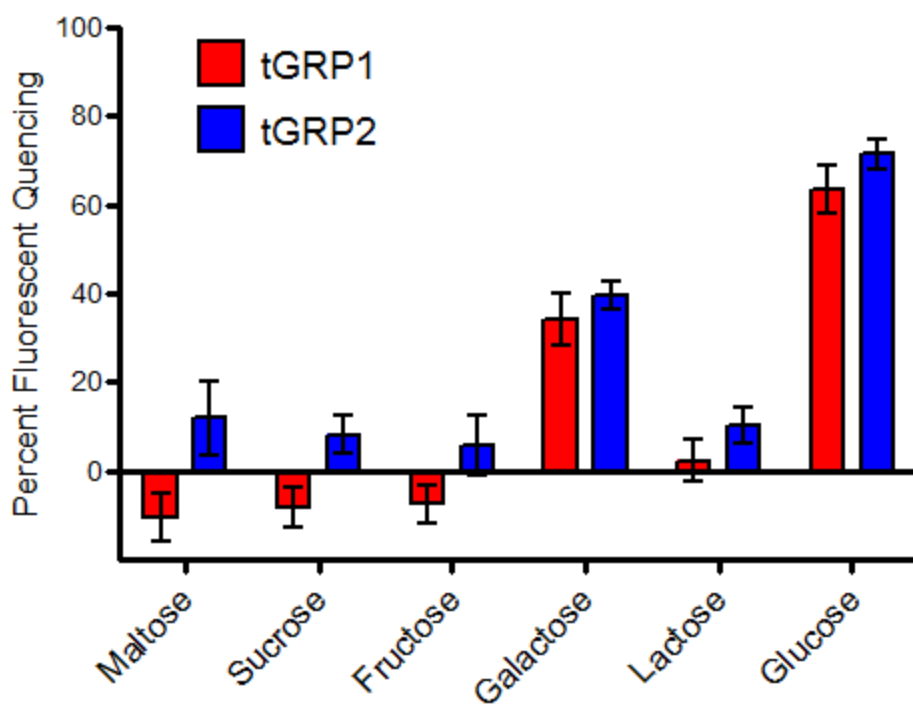


Figure 4.3. Selectivity study of tGRP1 and tGRP2 with selected sugars.

Samples for each sugar were prepared at a concentration of 100 mM in buffer and the experiment was carried out as described in Figure 1. The total fluorescence signal is quenched approximately 80% by glucose and 40% from by galactose. There was no significant response observed to maltose, sucrose, fructose, or lactose. Error bars denote +/- one standard deviation.

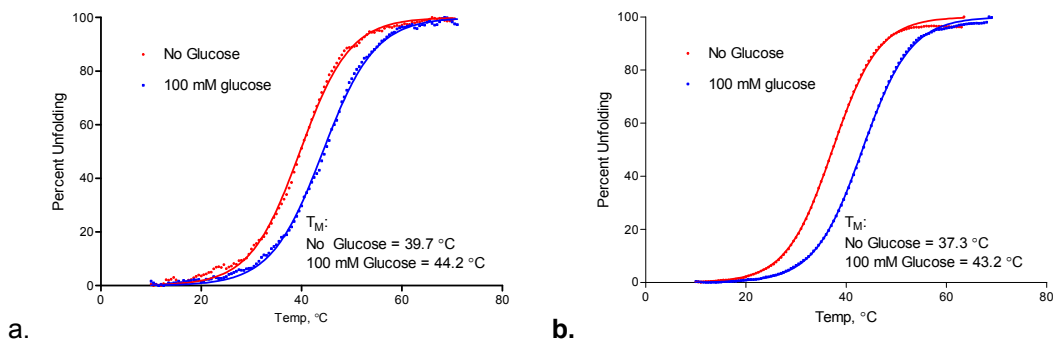


Figure 4.4. Thermal denaturation curves for tGRP1 (a.) and tGRP2 (b.) in the presence and absence of 100 mM glucose. Proteins were prepared at a concentration of 0.2 mg/mL in CD buffer (10 mM phosphate, 0.2 mM CaCl₂, pH 7.5) both in the absence of glucose and in the presence of 100 mM glucose. CD was monitored at 222 nm as the temperature was increased from 10 to 70 °C at a rate of 2 °C per min. The melting temperature for tGRP1 was 39.7 °C in the absence of glucose and 44.2 °C in the presence of glucose. The melting temperature for tGRP2 was 37.3 °C in the absence of glucose and 43.2 °C in the presence of glucose.

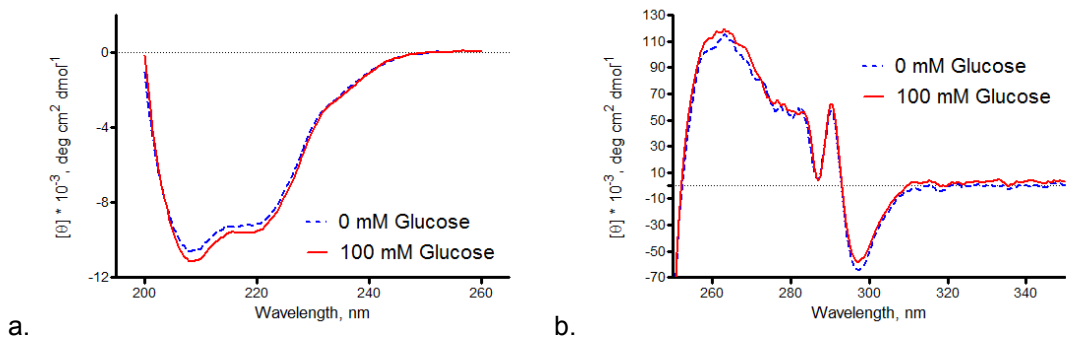


Figure 4.5. Far-UV (a.) and near-UV (b.) CD absorbance of tGRP1 in the presence and absence of 100 mM glucose.

Protein solutions and corresponding blanks were prepared at a concentration of 0.2 mg/mL in CD buffer in the presence and absence of glucose as described in Figure 4.3. The CD absorbance was measured from 260-200 nm at a pitch of 0.5 nm in continuous scanning mode at a scan speed of 20 nm/s for far-UV and 350-250 nm at a pitch of 0.5 nm and scan speed of 20 nm/min for near-UV measurements. Three accumulations were averaged for each sample at room temperature. The response for each blank was subtracted from the response for the corresponding sample and the resulting spectra are shown. characteristics of the proteins.

Further characterization of the tGRPs by circular dichroism revealed that, similar to native GBP, there was little change in the overall secondary structure. This is evident from the far-UV CD absorbance spectra for tGRP1 and tGRP2 shown in Figures 4.5b and 4.6 a., respectively. There is a slight change in the α -helix peak (approx. 210 nm and 220 nm) in the spectrum of tGRP1. This may be due to a small change in the folding of the helices in the vicinity of the truncated portion of the sequence. However, upon examining the individual spectra of the three data accumulations for each experiment, the differences may also be attributable to noise in the data.

The near-UV absorbance spectra (Figures 4.5 and 4.6 b) exhibit a small amount of change in the tertiary structure in the vicinity of the aromatic amino acids of the proteins upon glucose binding in the protein tGRP1. This is observed in the near-UV CD absorbance spectra shown in Figure 5b. The regions of the spectra which exhibit a slight difference correspond to phenylalanine (250-270) and tryptophan (280-300), while that corresponding to tyrosine (270-290) appears to remain unchanged. This is consistent with the known models for the conformational change for native GBP.[161] Examination of the local environments around these amino acids throughout the conformational change upon glucose binding gives some insight into the changes observed in the near-UV CD spectra. In particular, there is both a tryptophan and a phenylalanine residue present within the binding pocket (Figure 4.7), and both of these amino acids seem to undergo a change in solvent exposure upon

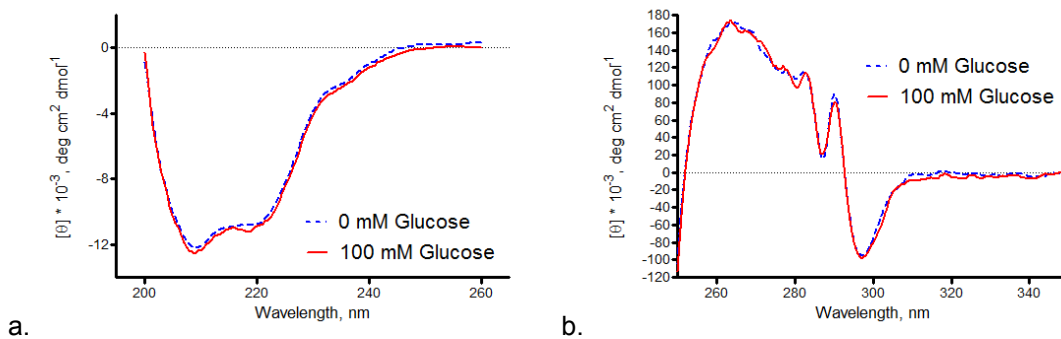


Figure 4.6. Far-UV (a.) and near-UV (b.) CD absorbance of tGRP2 in the presence and absence of 100 mM glucose.

Protein solutions and corresponding blanks were prepared at a concentration of 0.2 mg/mL in CD buffer in the presence and absence of glucose as described in Figure 4.3. The CD absorbance was measured from 260-200 nm at a pitch of 0.5 nm in continuous scanning mode at a scan speed of 20 nm/sec for far-UV and 350-250 nm at a pitch of 0.5 nm and scan speed of 20 nm/min for near-UV measurements. Three accumulations were averaged for each sample at room temperature. The response for each blank was subtracted from the response for the corresponding sample and the resulting spectra are shown.

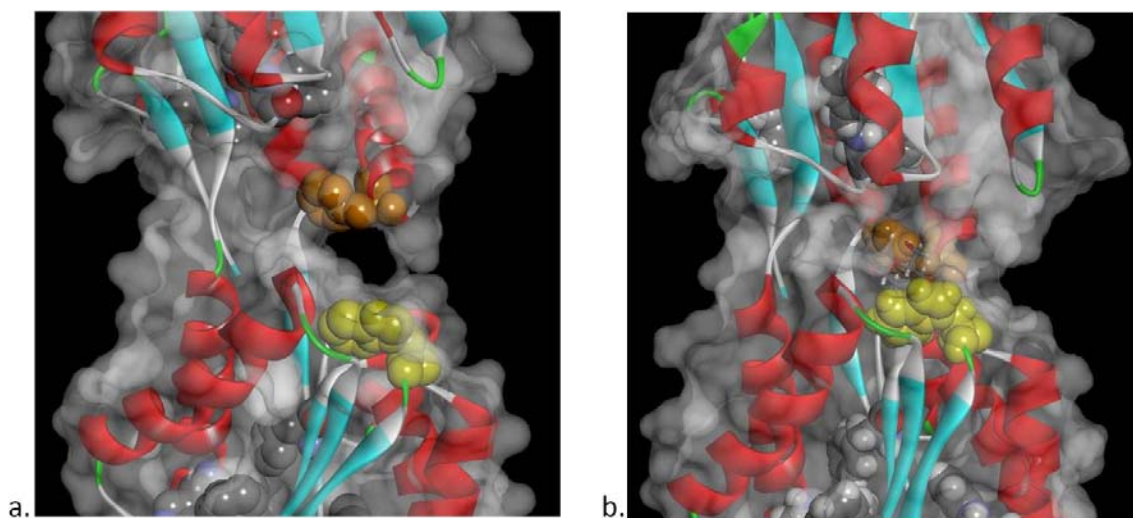


Figure 4.7. Modeled structure near the binding pocket in the absence (a.) and presence (b.) of glucose. The structures above were modeled using Discovery Studio 3.0 and coordinates provided in protein data bank (PDB) files 2FW0 and 2FVY, respectively. The tryptophan near the binding pocket is colored yellow, and the phenylalanine is colored orange.

glucose binding. None of the other aromatic amino acids undergo such a noticeable change upon ligand binding.

CONCLUSION

In conclusion, we present here a biosensor based upon truncated forms of GBP from *E. coli*. Previous work has shown full-length GBP to have a binding constant in the micromolar range, which is too low for the development of a glucose biosensing system at physiologically relevant concentrations. The apparent binding constant of the truncated proteins is shifted from the micromolar range, allowing for glucose determination in the physiologically relevant range. However, truncation of GBP also resulted in a decrease in thermal stability, decreasing the melting temperature for the protein when compared to its native form. Ongoing work in our laboratory aims to modify GBP through the incorporation of unnatural amino acids, which may further modify properties of the protein such as the binding constant and thermal stability. Additionally, the development of glucose recognition proteins based upon template proteins from thermophilic organisms should also yield more thermostable proteins.

CHAPTER FIVE

DESIGN AND CHARACTERIZATION OF A GLUCOSE BIOSENSOR BASED ON THE INTRINSIC FLUORESCENCE OF A TRUNCATED, THERMOSTABLE GLUCOSE RECOGNITION PEPTIDE

INTRODUCTION

The World Health Organization and others project that by 2050, as many as one-third of Americans may suffer from diabetes.[162] Among the contributing factors to this projection are poor lifestyle habits of the population, an aging population, and the increasing prevalence of other medical conditions as risk factors for diabetes. As a result of this worsening epidemic, a need exists for reliable technologies to monitor the physiological concentration of glucose in order to effectively manage the disease. Current-generation glucose monitoring systems rely on electrochemical methods for glucose detection, typically employing the enzyme glucose oxidase (GOx) in a mediator-based electrochemical sensor.[96, 149] Many of these devices are commercially available, both for single-point blood glucose measurement and for continuous glucose monitoring in interstitial fluid. While they have been very successful devices, they do suffer from some limitations. Among these limitations are interference from other electrochemically active molecules, oxygen-dependence, and poor performance in the hypoglycemic range.[96, 150]

As an alternative to electrochemical-based detection methods, various optical methods have been pursued for the detection of glucose in biological fluids.[72, 163] Among these are reagentless biosensing systems based on the binding ability of fluorescently-labeled glucose/galactose binding protein (GBP) from *E. coli* to glucose.[89] GBP is a well-characterized protein first identified in *E. coli*, which binds glucose in a binding cleft located between two lobes that are connected by a flexible hinge region consisting of three strands with random coil secondary structure. This binding results in a significant conformational change of the overall protein, bringing together the lobes by 8.5 Å.[159] By attaching environmentally-sensitive fluorophores to the protein, a change in the fluorescence signal corresponding to the glucose concentration can be observed. Extensive research in modifying native GBP and site-specifically attaching various fluorophores to it has resulted in the development of proteins capable of sensitive, reproducible detection of glucose at concentrations approaching those in the physiological range.[151, 153]

While work in the development of these fluorescent glucose biosensing systems has been ongoing for many years, no commercially-available sensing device has yet been developed. One factor limiting the development of a fluorescence-based glucose-sensing device is that the labeled GBP needs to remain stable for extended periods of time. To that end, the identification of GBPs from thermophilic organisms such as *Pyrococcus horikoshii*[164], *Thermus thermophilus*[165], and *Thermotoga maritima*[166] has opened new avenues for the development of more rugged, long-lived biosensing systems for glucose.

Proteins isolated from thermophilic organisms are remarkably stable with respect to extreme temperature, ionic strength, pH, and presence of chaotropic chemicals making them ideal for the development of biosensing systems that must be able to detect their target analyte in a sensitive and reproducible manner over long periods of time.[167]

In this work, we report the design and characterization of a glucose recognition peptide, tmGRP4, based upon a truncated form of the glucose binding protein (tmGBP) from the microorganism *Thermotoga maritima* (Figure 5.1). Previously, it has been shown that full-length tmGRP site-specifically labeled at various locations with a fluorophore is capable of detecting glucose with binding affinities ranging from 12.7 nM to 147.7 mM.[166] By studying truncated portions of the protein, we hypothesize that we can gain insights into the activity of the protein and identify portions that are required for ligand binding. This information will contribute to the further improvement and customization of the protein and the development of a minimized protein structure. Herein, we demonstrate the feasibility of using the intrinsic fluorescence of a truncated form of the protein resulting from a single tryptophan residue present in the protein's binding pocket as a means of detecting and quantifying glucose, thus eliminating the need for separate protein modification steps. Additionally, by monitoring changes in intrinsic fluorescence, we have been able to characterize the protein with respect to binding affinity and selectivity without perturbations in its structure, a potential drawback when proteins are modified with a fluorophore in a location near the binding pocket.

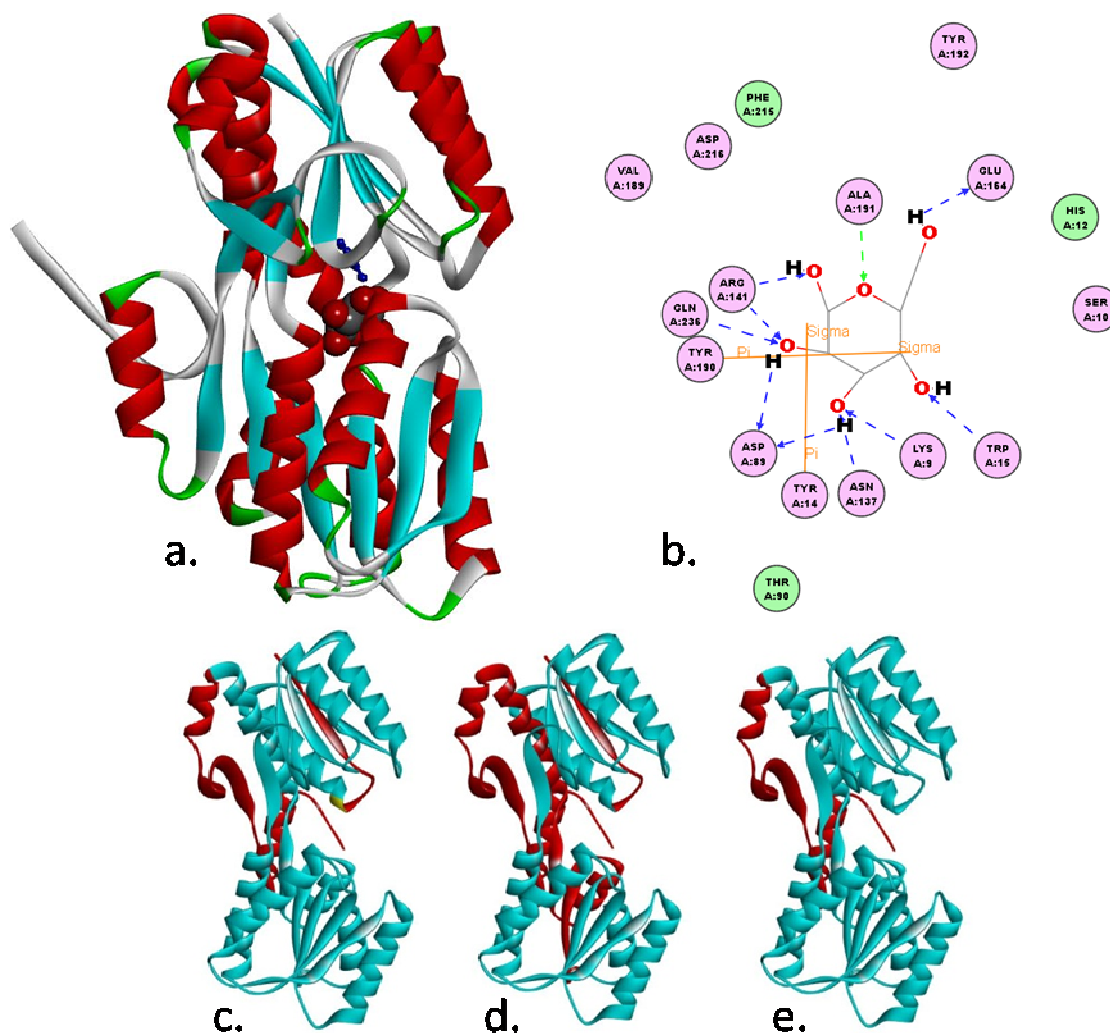


Figure 5.1. Protein structures of native tmGBP and truncated tGRPs. Native tmGBP (PDB ID: 2H3H) is shown with glucose in the binding pocket (a.) and a two-dimensional interaction map of amino acids in the binding pocket is shown (b.). Truncated tGRP4 (c.), tGRP5 (d.), and tGRP6 (e.) are shown with truncated portions of the protein colored red. The protein renderings and amino acid interaction map were prepared using Discovery Studio 3.0.

EXPERIMENTAL PROCEDURES

Reagents. Oligonucleotide primers were purchased from Operon Biotechnologies (Huntsville, AL). *Thermotoga maritima* genomic DNA was purchased from ATCC (Manassas, VA). Ampicillin, chloramphenicol, glucose, sodium ascorbate, D(+)-raffinose, cholesterol, D-Lyxose, L(+)-arabinose, D(+)-maltose, lactose, 2-amino-2-hydroxymethyl-propane-1,3-diol (tris base), β -mercaptoethanol, and agarose were purchased from Sigma (St. Louis, MO). Luria-Bertani (LB) broth and agar, sodium chloride, sucrose, sodium phosphate monobasic, sodium phosphate dibasic and glycine were purchased from Fisher Scientific (Fair Lawn, NJ). Restriction enzymes XhoI and BamHI and T4 DNA ligase were purchased from Promega (Madison, WI). Ni-NTA agarose resin, QIAquick gel purification kit, and QIAprep DNA isolation kit were purchased from Qiagen (Valencia, CA). QuickChange II site-directed mutagenesis kit and pfuUltrall DNA polymerase were purchased from Stratagene (La Jolla, CA). Imidazole and guanidine hydrochloride were purchased from J.T. Baker (Phillipsburg, NJ). D(+)-galactose was purchased from Acros Organics (Geel, Belgium). Sodium dodecyl sulfate (SDS) was purchased from Curtin Matheson (Houston, TX). Tris(2-carboxyethyl)phosphine (TCEP) and Rosetta 2 (DE3) cells were purchased from EMD (Gibbstown, NJ). Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). TOP10F' cells were purchased from Invitrogen (Carlsbad, CA). The vector pET21a(+) was purchased from Novagen (Darmstadt, Germany). D(+)-fructose was purchased from Spectrum (Gardena, CA).

Apparatus. Polymerase chain reactions (PCR) were performed using an Eppendorf Mastercycler Personal Thermocycler (Hauppauge, NY). Electrophoresis of DNA was carried out using an FB105 Fischer Biotech Electrophoresis Power Supply (Pittsburg, PA). DNA gels were visualized using a UV Transilluminator platform from UVP (Upland, CA). Optical density measurements were taken using a Spectronic 21D from Milton Roy (Ivy Land, PA). Cells were lysed using a 550 Sonic Dismembrator from Fisher Scientific (Pittsburg, PA). Cell cultures were grown by incubating bacteria in a Forma Scientific Orbital Shaker (Fairlawn, NJ). All centrifugations were carried out using a Beckman J2MI centrifuge (Palo Alto, CA). Proteins were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN TGX 4-20% SDS-PAGE gels purchased from Bio-Rad (Hercules, CA). Fluorescence measurements were obtained using a QuantaMaster 40 Spectrofluorometer from PTI (Birmingham, NJ). Circular dichroism experiments were performed using a BioLogic MOS 450/PMS 450 Spectrometer from BioLogic (Claix, France).

Cloning of Glucose Recognition Peptides (tGRPs) into Expression Plasmid.

The gene for GBP from *T. maritima* was isolated by PCR from purchased genomic DNA using the primers tmGRP-21aXhoI (5'-GGTGGTCTCGAGGAATTTTATTGGAATTCCG-3') and tmGRP-21aBamHI (5'-GGTGGTGGATCCCTCACCATAGGTGTTATCGG-3') with the restriction sites XhoI and BamHI underlined, respectively. The PCR reaction was carried out using pfuUltraII DNA polymerase with an initial denaturation of 95 °C for 2 min.

Next, 30 cycles of a denaturation step at 95 °C for 30 s, and annealing step at 63 °C for 20 s, and an elongation step at 72 °C for 30 s were carried out. A final elongation step of 72 °C for 3 min completed the PCR reaction. The resulting products were analyzed by agarose gel electrophoresis for correct size and purity, and the appropriately sized band (915 bp) was excised from the gel and purified using the QIAquick gel extraction kit.

Following PCR product purification, the product along with the expression vector pET21a(+) were digested with restriction enzymes XhoI and BamHI. Digestion was carried out per instructions provided with the restriction enzymes. Following digestion, the products were analyzed by agarose gel electrophoresis. Next, a gel slice of digested PCR product was combined with a gel slice of digested expression vector and co-purified using the QIAquick gel extraction kit. To the purified DNA, T4 ligase and T4 ligase buffer (10x) were added, and the ligation reaction was allowed to complete overnight at room temperature. The resulting ligated DNA was transformed into TOP10F' cells following standard transformation procedures. Colonies resulting from the transformation were analyzed for the presence of the correct insert by overnight growth in LB media supplemented with ampicillin (100 µg/mL) and digestion of the purified DNA with restriction enzymes XhoI and BamHI as above. Glycerol stocks were made of those colonies which appeared correct, and the isolated plasmid, tmGRP-pET21a(+), was sent for DNA sequencing for confirmation.

In order to introduce a cysteine at position 135, site-directed mutagenesis was carried out using the QuickChange II site-directed mutagenesis kit. The

mutagenesis primers used were tmGRP-mut-135A/C (5'-ACGGGTTCACTGACATGTATGAACTCCCTTCAG-3') and tmGRP-135A/C-rev (5'-CTGAAGGGAGTTCATACATGTCAGTGAACCCGA-3'). The reaction mixture was prepared per the kit instructions. An initial denaturation step was performed at 95 °C for 30 s. Following this, 30 cycles of a denaturation step at 95 °C for 30 s, an annealing step of 55 °C for 1 min, and an elongation step of 68 °C for 5 min were completed. A final elongation step was performed at 68 °C for 5 min. The resulting products were analyzed for purity and correct size by agarose gel electrophoresis. The resulting mutated plasmid was transformed into TOP10F' cells following standard transformation protocols. Following the transformation, colonies were analyzed for the presence of the correct plasmid by growth overnight in LB broth supplemented with ampicillin (100 µg/mL). From the overnight cultures, the plasmid DNA was isolated and digested as above with XhoI and BamHI and analyzed by agarose gel electrophoresis to confirm the presence of the insert and expression vector bands. Glycerol stocks were prepared of appropriate colonies, and the isolated plasmid, tmGRP135C-pET21a(+), was sent for DNA sequencing for confirmation.

For the preparation of truncated glucose binding proteins from *T. maritima* (tmGRP4), PCR was performed using the above prepared plasmid, tmGRP135C-pET21a(+), as template DNA. The final truncated proteins were designed to consist of amino acids 14-254 (tGRP4), 14-206 (tGRP5) and 1-254 (tGRP6) of the native protein. A PCR reaction was carried out for truncation of the tmGRP135 gene using the primers tmGRP4-fwd-BamHI (5'-

GGTGGTGGATCCTACTGGTCACAGGTAGAACAAGGT-3') and tmGRP4-rev-XhoI (5'-GGTGGTCTCGAGCTTGTTTCATCAGATAAAGAACTGT-3') for the amplification of tGRP4, the primers tmGRP4-fwd-BamHI and tmGRP5-rev-XhoI (5'-GGTGGTCTCGAGTTTTCCAGCATTTCACCCACGAG-3') for the amplification of tGRP5, and the primers tmGRP6-fwd-BamHI (5'-GGTGGTGGATCCCTCCACCATAGGTGTTATCGGAAAA-3') and tGRP4-rev-XhoI for the amplification of tGRP6. PCR reaction parameters were identical as above for the preparation of tmGRP-pET21a(+). Following the PCR reaction, the products were analyzed by agarose gel electrophoresis. DNA bands for tGRP4 (723 bp), tGRP5 (579 bp), and tGRP6 (759 bp) were excised and purified using the QIAquick kit. After DNA isolation, each product, along with expression vector pET21a(+) were digested with restriction enzymes XhoI and BamHI as above. The digested DNA was purified by agarose gel electrophoresis, and the appropriate DNA bands were excised from the gel. In separate tubes, excised DNA of each truncated product was combined with excised DNA for the expression vector, and co-purified using the QIAquick kit. To the purified DNA, T4 DNA ligase and T4 ligase buffer (10x) were added, and the ligation reaction was incubated overnight at room temperature. After ligation, the resulting plasmids, tGRP4-pET21a(+), tGRP5-pET21a(+), and tGRP6-pET21a(+) were transformed into Rosetta2(DE3) cells for protein expression. Colonies from these transformations were grown overnight in LB broth supplemented with ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL), preserved as glycerol

stocks, and plasmid DNA was isolated as before and sent for sequencing for confirmation.

Recombinant Protein Expression and Purification. For the expression of tGRP4, an overnight culture of tGRP4-pET21a(+) in Rosetta2(DE3) was grown in LB media supplemented with ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL). The following day, expression cultures were grown (2 x 500 mL) in LB broth supplemented with ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) at 37 °C to an OD₆₀₀ of approximately 0.5. Protein expression was induced by the addition of IPTG to a final concentration of 1.0 mM, and the culture was grown overnight at 37 °C.

In order to harvest the cells after protein expression, the culture was centrifuged at 12,000 x g, at 4 °C, for 20 min. Cells were resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by pulsed sonication (10 s on, 10 s off, 10 min total sonication). Following cell lysis, the lysate was centrifuged at 12,000 x g, at 4 °C, for 20 min, to remove debris. The supernatant was transferred to a fresh centrifuge tube, 1.0 mL of Ni-NTA agarose resin was added, and the solution was incubated at 4 °C for 1 h. The solution was then applied to a gravity-flow column, and the flow-through fraction was collected. The resin was then washed with 10 mL of wash buffer 1 (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0), then 3 mL of wash buffer 2 (50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole, pH 8.0), and finally 3 mL of wash buffer 3 (50 mM NaH₂PO₄, 300 mM NaCl, 100 mM imidazole, pH 8.0). The wash fractions were collected in separate tubes. Finally, the protein was eluted

from the column in six 1.0 mL fractions of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). Following purification, the products were analyzed for purity by SDS-PAGE. Elution fractions containing purified protein were combined in an amber glass vial and stored at 4 °C. Protein concentrations were determined using method A detailed by Greenfield *et al.*[156]

Fluorescence Measurements of GRPs. All fluorescence measurements were carried out in assay buffer (20 mM phosphate, 20 mM NaCl, 1.0 mM TCEP, pH 7.0) at a protein concentration of 1.0 x 10⁻⁶ M. Glucose standards were prepared in assay buffer and stored at 4 °C. Tryptophan fluorescence was excited at 278 nm and emission was measured at 340 nm. For excitation and emission scans, instrument settings were as follows: excitation and emission slit widths were set at 1 nm and 3 nm respectively, monochromator step size was set at 0.5 nm, integration time was 0.5 s, 3 scans were averaged for each acquisition. For glucose assays, a time-based acquisition program was established with the same excitation and emission wavelengths as above, and fluorescence intensity was averaged over 2.5 s with a single reading every 0.5 s. Apparent binding constants were determined using the statistical analysis software GraphPad 5.0. Briefly, the fluorescence intensity was plotted versus glucose concentration and a sigmoidal response curve was fitted to the data following Equation 1:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC_{50}) - x \times Hillslope}} \quad \text{Equation 1}$$

In Equation 1, the values for Top and Bottom are determined by the plateau regions resulting from the highest and lowest concentration data points,

respectively. The Hill slope is variable and dependent upon the slope along the curve calculated about each data point. The $\log EC_{50}$ is the logarithm of the concentration that results in a change in signal halfway between the Top and Bottom values, or when half of the protein binding sites are occupied. For this reason, the apparent binding constant can be derived from the $\log EC_{50}$ value.

Determination of Protein Thermal Stability. Protein thermal stability was investigated by the measurement in intrinsic tryptophan fluorescence as a function of temperature and the concentration of the chaotropic agent guanidine hydrochloride. Briefly, the same protein concentration was used for each experiment. The instrument settings were also kept the same as described above. Fluorescence was measured at the wavelengths used above throughout a temperature ramp from 20 °C to 90 °C at a rate of 5 °C per minute with stirring. An initial hold time of 180 s at 20 °C was used prior to the temperature ramp, and the linear decay of fluorescence was extrapolated throughout the total experiment time and subtracted from the temperature ramp fluorescence curve in order to account for the effects of photobleaching throughout the experiment. Samples were measured both in the absence of glucose, and with a glucose concentration of 1.0 M. Additionally, the concentration of guanidine hydrochloride was varied from 0.0 M to 4.5 M.

Measurement of Far- and Near-UV Circular Dichroism (CD) Spectra. To investigate protein conformational changes upon glucose binding, both far- and near-UV circular dichroism spectra were measured. For far-UV CD, the proteins were evaluated at a concentration of 0.2 mg/mL in CD buffer (20 mM phosphate,

20 mM NaCl, pH 7.0) in a quartz cuvette with a pathlength of 1.0 mm. Instrument settings were as follows: wavelength range was 180-260 nm, scan repeat was 4, acquisition duration was 20 s, and shutter mode was always open. Samples were measured in the absence of glucose, and with a glucose concentration of 100 mM. Corresponding blanks were measured with the same glucose concentrations in buffer, with no protein present, and subtracted from the protein CD data. For near-UV CD, the proteins were evaluated at a concentration of 1.0 mg/mL in CD buffer in a quartz cuvette with a pathlength of 1.0 cm. Instrument settings and sample preparation were as above for far-UV CD, except the wavelength range was 250-350 nm.

Determination of Protein Specificity. Protein specificity was determined for selected analytes. Sample solutions of protein in assay buffer were prepared in a similar manner as for glucose assays. To these protein solutions, the selected analytes were added, in triplicate, and the fluorescence intensity was measured. Analytes were added to the protein solution at a final concentration of 1.0 mM with the exception of cholesterol, and ascorbate, which were added at a final concentration of 10 and 50 μ M, respectively.

RESULTS AND DISCUSSION

In this work, we report the design of a truncated form of the glucose binding protein from *Thermotoga maritima*. By studying truncated versions of the full-length protein, we have gained some insight into portions of the protein which

are necessary for proper folding and activity. Specifically, it was observed that tGRP5, which lacks 113 out of 305 amino acids of full-length tmGRP was unable to properly fold and/or respond to glucose (data not shown). However tGRP4, which lacks 65 amino acids, maintains a response to glucose. This suggests that some of the amino acids removed in tGRP5 must be essential for protein folding and glucose interaction. Previously published strategies for engineering glucose binding proteins to elicit an optical response have relied on fusion with a suitable bioluminescent or fluorescent reporter protein[72, 168] or the site-specific incorporation of a cysteine residue and subsequent covalent attachment of environmentally-sensitive organic fluorophores.[89, 151] The engineered protein in this work, tGRP4, contains a single tryptophan residue that resides in the binding cavity of the protein and interacts directly with glucose through a hydrogen bonding interaction (figure 5.1). When tGRP4 is bound to glucose, this tryptophan shows a concentration-dependent enhancement of intrinsic tryptophan fluorescence resulting in a total fluorescence enhancement of approximately 9% at maximum signal intensity and an apparent binding constant of 3.0×10^{-6} (figure 5.2). Continuous, time-based measurements of tGRP4 in solution also showed that the total change in fluorescence is observed within 5 s of the addition of glucose (data not shown).

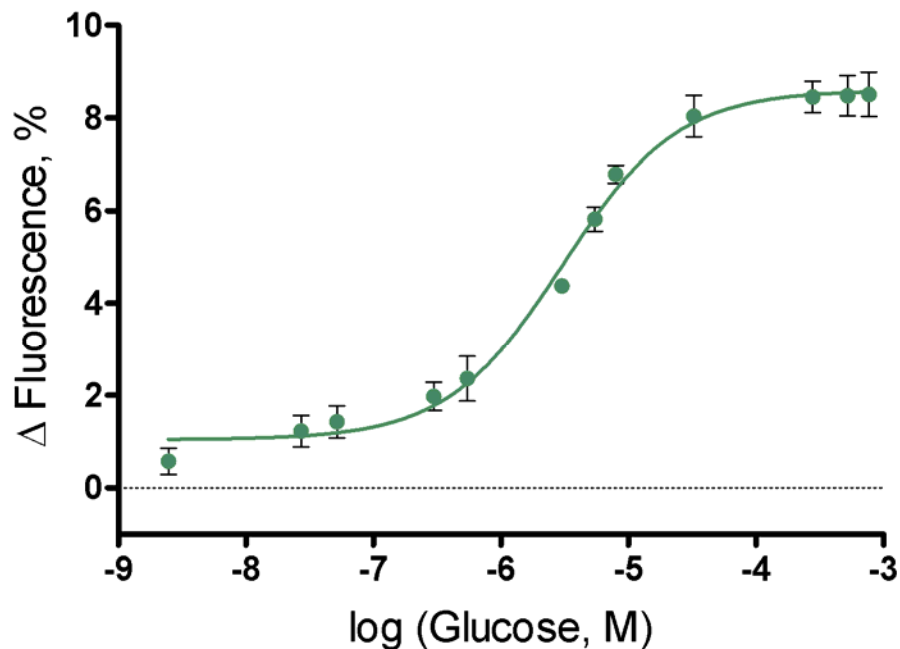


Figure 5.2. Calibration curve of tGRP4 in response to glucose. The percent enhancement of the intrinsic tryptophan fluorescence intensity with respect to glucose concentration is shown above. tGRP4 was added to 2.0 mL of assay buffer at a final concentration of 1.0×10^{-6} M in a quartz cuvette with a path length of 1 cm. Glucose was added from standard solutions in aliquots of $0.5 \mu\text{M}$ resulting in the final concentrations above. Data represent an average of 3 replicate measurements with standard deviations depicted with error bars (some error bars obscured by datapoints). A sigmoidal-response curve was fitted using GraphPad Prism 5.0 software, and an apparent binding constant of 3.0×10^{-6} M was calculated based on the curve.

Previously developed biosensors based on GBP from *E. coli* have been shown to respond to galactose as well as glucose with a slightly higher binding affinity latter.[89] Work in our laboratory has demonstrated that truncating the native protein structure for GBP from *E. coli* alters the binding affinity and response of the protein. To investigate whether the selectivity of tGRP4 is altered compared to full-length tmGBP, we measured the response of the protein to several other sugars as well as cholesterol and ascorbate, which have been shown to act as interfering species in electrochemically-based glucose sensors. The compounds were investigated at the following concentrations: ascorbate was added at a concentration of 50 μM , cholesterol was added at a concentration of 10 μM , and all sugars were added at a concentration of 1 mM. As shown in figure 5.3, the addition of maltose, a disaccharide of glucose, results in an enhancement of fluorescence that is indistinguishable from glucose. However, physiologically, maltose is rapidly metabolized to glucose. Additionally, there is a significant quenching in fluorescence resulting from the addition of ascorbate. This quenching effect is also observed in solutions of tryptophan (data not shown) indicating that the effect is not due to the binding of ascorbate to the protein, but rather to quenching of tryptophan fluorescence by ascorbic acid.

To investigate this effect, we performed experiments to determine the effects of various physiologically-relevant concentrations of ascorbic acid on the response of the protein (Figure 5.4). Indeed, as the concentration of ascorbic acid was increased, the raw fluorescence measured decreased. However, it was observed that the addition of glucose still resulted in fluorescence enhancement,

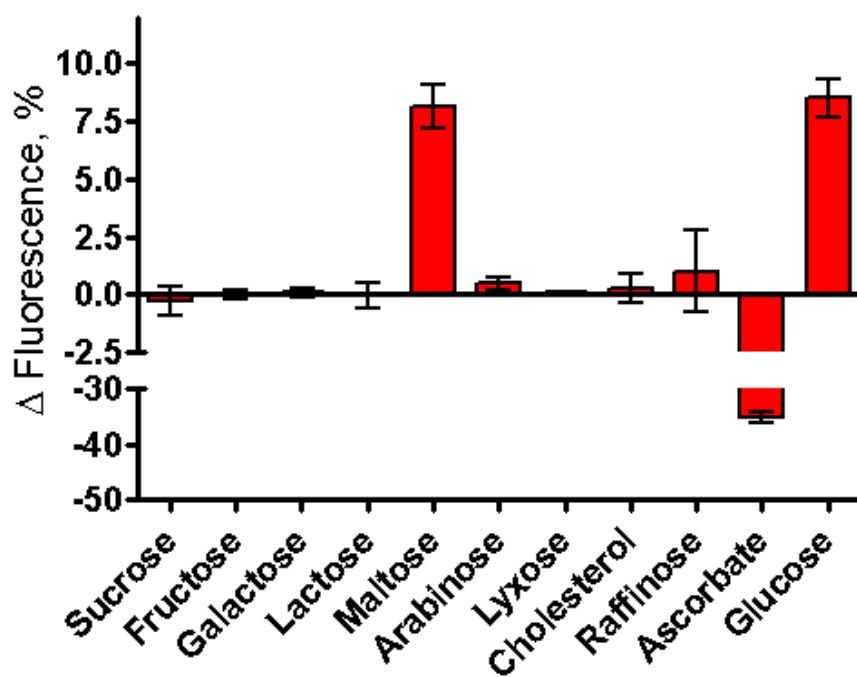


Figure 5.3. Selectivity study of tGRP4. Each compound was added at a final concentration of 1.0 mM in triplicate, except ascorbate which was added at 50 μ M and cholesterol, which was added at 10 μ M. The response of maltose was indistinguishable from that of glucose, and ascorbate demonstrated a dramatic quenching effect of tryptophan fluorescence at the concentration above.

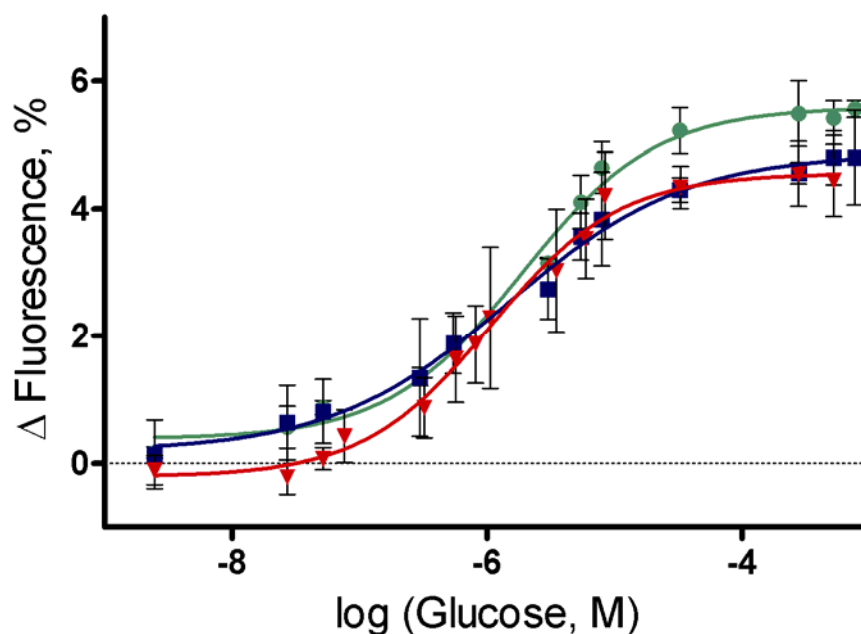


Figure 5.4. Glucose calibration curves in the presence of ascorbate. Glucose assays were performed at 0 μM (\blacktriangledown), 10 μM (\bullet), and 100 μM (\blacksquare) of ascorbate. While the percent enhancement of tryptophan fluorescence was maintained, the raw signal was decreased as the concentration of ascorbate was increased. Data represent an average of 3 replicate measurements with standard deviations depicted with error bars (some error bars obscured by data points). A sigmoidal-response curve was fitted using GraphPad Prism 5.0 software,

and the percent enhancement of fluorescence was virtually identical to that observed in the absence of ascorbic acid. Because of this, it is believed that a biosensing system which employs the intrinsic fluorescence of tGRP4 would still be feasible if a standard addition approach was used to eliminate the interference from ascorbic acid.

In the development of a commercially-viable device, an important parameter for any blood glucose monitoring system is sensor lifetime, especially for continuous glucose monitors (CGMs). Sensor lifetimes are limited by several factors, most prominently an immune response to the implanted sensor as a foreign body.[169] There are only a few FDA-approved CGMs currently on the market, and most of these have sensor lifetimes of 3-5 days, with the longest being 7 days. To test the lifetime of tGRP4, we compared the response of fresh protein from storage at 4 °C to that of protein stored long-term at 37 °C. The results of this study, shown in figure 5.5, reveal that virtually no loss in tGRP4 activity is observed within 72 h. The protein is still capable of glucose detection for as long as 5 days, albeit at a decreased response.

To further investigate the thermal stability of tGRP4, glucose assays were carried out at temperatures ranging from 37 °C to 60 °C (Figure 5.6). The importance of protein stability to high temperatures related not just to physiological measurements, but also to transport and storage of proteins that

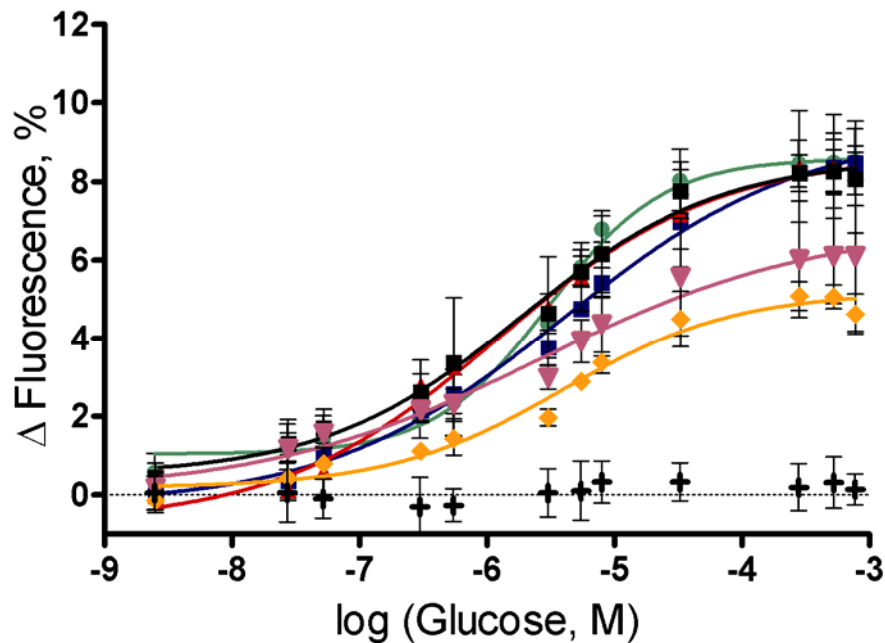


Figure 5.5. Effect of long-term storage at 37 °C on assay performance. Glucose assays were completed with protein solution fresh from storage at 4 °C (●), and after protein storage at 37 °C for 24 h (■), 48 h (▲), 72 h (■), 96 h (▼), 120 h (◆), and 168 h (+). Protein activity was maintained through 72 h, at which time the percent fluorescence enhancement began to decrease. No glucose response was observed beyond 120 h. Data represent an average of 3 replicate measurements with standard deviations depicted with error bars (some error bars may be obscured by data points). A sigmoidal-response curve was fitted using GraphPad Prism 5.0 software,

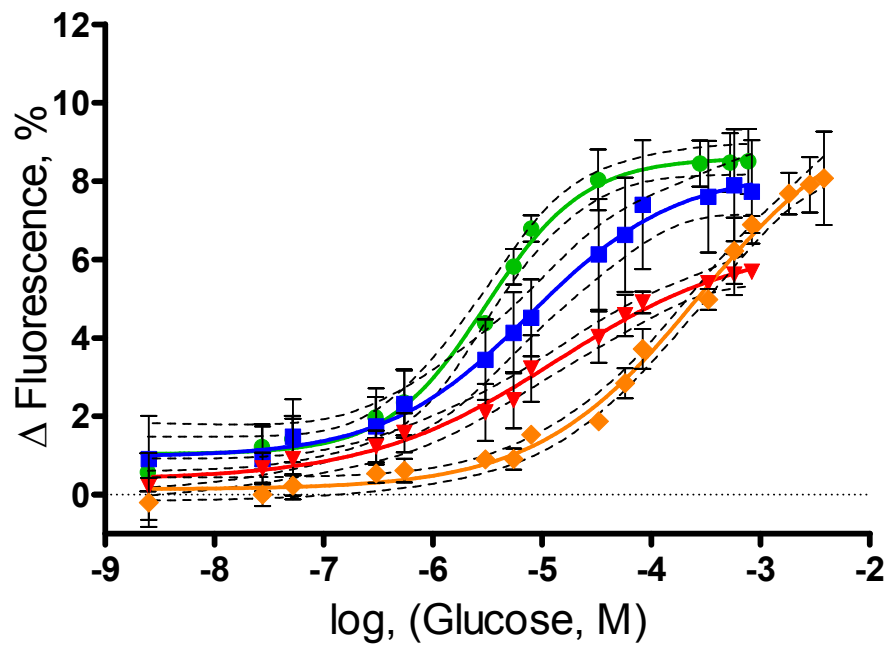


Figure 5.6. Effect of temperature on glucose response. Glucose assays were performed at 37 °C (●), 44 °C (■), 50 °C (▼), and 60 °C (◆). Data represent an average of 3 replicate measurements with standard deviations depicted with error bars (some error bars may be obscured by data points). A sigmoidal-response curve was fitted using GraphPad Prism 5.0 software.

need to be used in devices and as diagnostic tools. Proteins with improved thermal stability tend to have increased tolerance to elevated temperatures during measurement and improved shelf-life at non-ideal storage and transport conditions (i.e., tropical or desert environments). This improved tolerance reduces the need for special storage conditions, making devices based upon these proteins more amenable to use in extreme environments. As expected for a thermostable protein, activity was maintained at higher temperatures. As the temperature was increased further to 60 °C, the response curve was shifted to an increased apparent binding affinity of 2.6×10^{-4} M. For a thermostable protein, it would be expected that binding would be stronger at a higher temperature. This observed shift in binding affinity is likely the result of a decreased rigidity in the protein structure of the truncated protein as compared to the full-length protein. As the overall flexibility of the protein structure is increased, the binding of glucose within the binding cavity likely becomes less favorable. As the temperature increased, a decrease in absolute fluorescence intensity was also observed. This can likely be attributed to increased collisional quenching as the flexibility of the protein is increased and the exposure of tryptophan to the solvent increases.

The stability of the protein structure was further explored by measuring the fluorescence intensity of the tryptophan residue as the temperature was increased from room temperature to 90 °C in the presence of the chaotropic agent, guanidine hydrochloride. Guanidine was added to shift the melting transition of the protein to a temperature that is experimentally relevant, since the

protein in the absence of chaotropic agents has a melting temperature well above the boiling point of water. As mentioned above, tGRP4 contains a single tryptophan residue located within the binding cavity, in the hydrophobic interior of the protein. As chemical and thermal stresses are placed on the protein, its secondary and tertiary structural features should begin to unravel as the protein denatures. This should result in the buried tryptophan residue becoming more exposed to the aqueous environment surrounding the protein, and a decrease in measured intrinsic tryptophan fluorescence. It has also been previously shown that the presence of glucose contributes to an increased stability of the protein structure of GBPs, resulting in an increase in thermal- and chemical-stability.[160, 166] Figure 5.7 shows the normalized tryptophan fluorescence as a function of temperature with various concentrations of guanidine present. As shown in figure 5.7a, the melting transition is observed to begin around 70 °C in the absence of glucose and around 80 °C in the presence of glucose at a guanidine concentration of 1.0 M, and the protein never reaches a completely unfolded state, as indicated by the continuous decrease in fluorescence. At 3.0 M guanidine (figure 5.7b), the protein begins to unfold at a temperature just above 40 °C in the absence of glucose, and begins to reach a completely unfolded state at around 70 °C. In the presence of glucose, protein structure is maintained and the melting transition is again observed to begin at approximately 80 °C. Finally, at a concentration of 4.5 M guanidine (figure 5.7c) and in the absence of glucose, the melting transition begins just above 20 °C, indicating a highly denaturing environment, however, denaturation is still incomplete until a

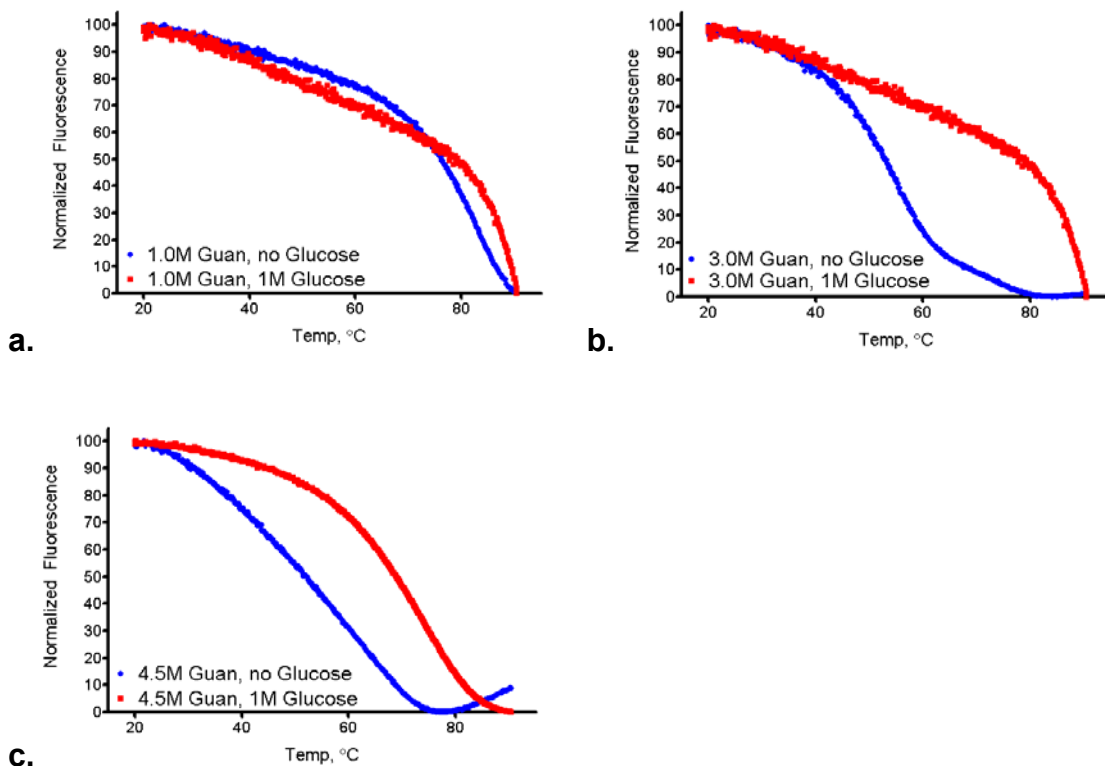


Figure 5.7. Thermal and chemical denaturation of tGRP4. Assay solutions were prepared in the absence and presence of glucose with various concentrations of guanidinium hydrochloride (Guan). While measuring tryptophan fluorescence, the temperature was ramped from 20-90 °C. Protein melting was observed as the tryptophan fluorescence is quenched. As the concentration of guanidinium was increased, the melting transition shifted to lower temperatures. As shown by the increased melting transition temperatures, the presence of glucose increased the stability of the protein structure.

temperature of approximately 75 °C. In the presence of glucose at this guanidine concentration, denaturation does not begin until approximately 60 °C, and the protein is not completely disordered until over 80 °C.

In order to investigate the conformational change which occurs in tGRP4 upon glucose binding, the far- and near-UV CD spectra were measured in the absence and presence of glucose. As shown in figure 5.8a, the far-UV CD spectrum, which reveals changes in protein secondary structure, showed little change in response to glucose. This is consistent with available structural data for full-length tmGBP. Near-UV spectra, on the other hand, provides information on changes in the tertiary structure in the environment around the aromatic amino acids phenylalanine (250-270 nm), tyrosine (270-290 nm), and tryptophan (280-300 nm). As shown in figure 5.8b, there is a broad peak centered around 275 nm that is somewhat increased in the presence of glucose. This is likely attributed to a tyrosine located on the edge of the binding pocket that is believed to experience a change in solvent exposure as a result of the conformational change.

CONCLUSION

In this work, we have developed a glucose biosensor using a thermally stable protein, tGRP4, which is based on a truncated form of GBP from the hyperthermophilic organism, *Thermotoga maritima*. Many previous glucose

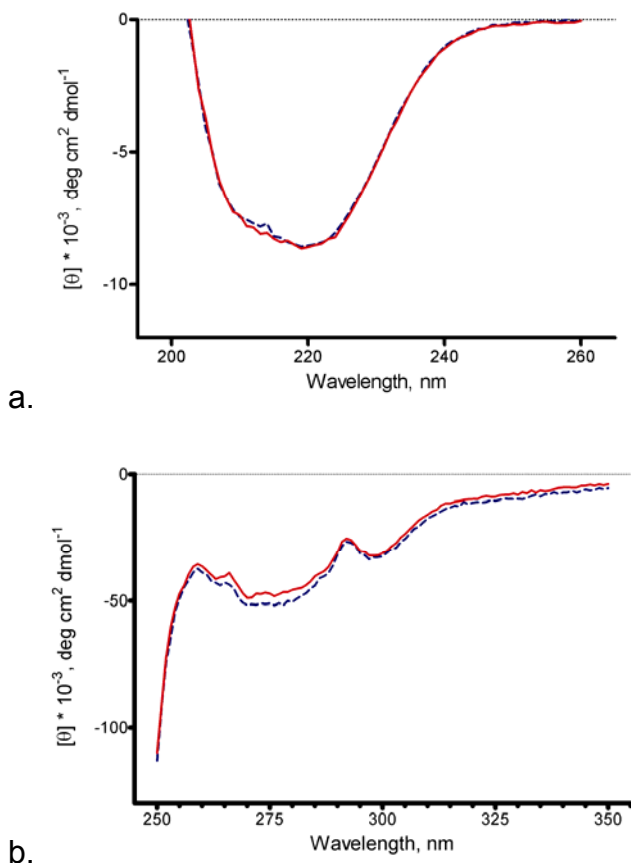


Figure 5.8. Far-UV (a.) and near-UV (b.)CD absorbance of tGRP4 in the presence and absence of 100 mM glucose. Protein solutions and corresponding blanks were prepared at a concentration of 0.2 mg/mL in CD buffer containing 0 mM (---) or 100 mM (—) glucose as described in figure 5.3. The CD absorbance was measured from 200-260 nm for far-UV and 250-350 nm for near-UV measurements. Four accumulations were averaged for each sample at room temperature. The response for each blank was subtracted from the response for the corresponding sample and the resulting spectra are shown.

biosensing systems based on glucose binding proteins rely on the covalent attachment of a small fluorophore to a specific position within the protein structure. The work presented herein instead relies on the intrinsic fluorescence of a tryptophan residue located within the binding cavity of the protein, negating the time and expense of the extra labeling steps required for fluorophore-labeled proteins. This approach could be applied to other protein-based sensing systems through the introduction of a unique tryptophan within a protein's structure. We have shown that tmGRP can withstand the removal of 65 amino acids (over 20% of the total protein) while still maintaining binding activity. This provides insight into developing a minimized protein structure, which may be more amenable to customizability. We have also demonstrated several desirable properties of a CGM system such as a sensor lifetime on the order of 3-5 days, maintained sensor performance under extreme conditions, and accurate detection of glucose which was both reproducible, and selective. The improved thermal stability gained from using a hyperthermophilic variant of GBP provides the advantages of a rugged protein that is more tolerable of extreme conditions such as temperature both during use in a biosensing system as well as during storage and transport. We envision that integration of the engineered protein into a fiber-optic based biosensing system will result in the design and development of long-lived practical optical glucose monitoring platforms and devices capable of performance at physiological temperatures and pH, and that are selective to glucose over a wide variety of sugars.

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CHAPTER SIX

CONCLUSIONS AND FUTURE PERSPECTIVES

Nature has spent billions of years evolving mechanisms by which organisms can sense and respond selectively to stimuli in their surrounding environment. This degree of adaptation has allowed organisms to identify and transport needed nutrients into cells, to remove waste products that would otherwise accumulate to toxic concentrations within cells, and to defend against exposure to harmful toxicants. The biological sensing tools that nature has developed to accomplish these tasks have been discussed extensively in this dissertation and include regulatory proteins that recognize small effector molecules and trigger the expression of appropriate genes and chemotaxis proteins that actively transport small molecules into and out of cells. It has been the goal of this work to exploit these naturally occurring biosensing systems and engineer them to serve as sensitive, selective, reproducible, and rugged systems which can be employed in the detection of small molecules of interest, including both biologically and physiologically relevant compounds. Additionally, we have gained some insight into the relationship between the structure of the various sensing proteins studied and their relevant analytical response characteristics.

Chapter two presented a whole cell sensing system for hydroxylated polychlorinated biphenyls (OH-PCBs). These compounds are metabolites of PCBs and can serve as a biomarker of PCB exposure. By employing the

regulatory protein, HbpR, from the bacterium *Pseudomonas azelaica*, we were able to construct a biosensing system capable of detection OH-PCBs at concentrations down to the nanomolar range. In this strategy, the expression of the reporter protein, bacterial luciferase, was put under the regulatory control of HbpR. Chapter three presents a protein-based sensing system based upon HbpR. In this work, the effector binding A-domain of HbpR (HbpR-A) was isolated and labeled at cysteines in the structure with an environmentally-sensitive fluorophore, 5-[2-[(2-Iodo-1-oxoethyl)amino]ethylamino]-1-naphthalenesulfonic acid (IAEDANS). Upon analyte binding, likely due to a conformational change in HbpR-A, a change in the fluorescence intensity observed that could be correlated to OH-PCB concentration. A limiting factor in the development of the protein-based biosensor was the lack of structural information available for HbpR. Serendipitously, our protein was responsive to OH-PCBs when labeled with IAEDANS. Determination of the three-dimensional structure of HbpR would allow a more rational approach to improving the response of the sensing system. For instance, by replacing or removing any cysteines which are not affected by the conformational change, the background fluorescence could be reduced, which should result in improved detection limits. Additionally, identification of amino acids present in the binding pocket of the protein and elucidation of the specific interactions between these amino acids and the OH-PCBS should provide the opportunity to rationally alter these interactions to improve the selectivity of the protein which could eventually allow us to tailor the protein such that we can preferentially detect some OH-PCBs

(e.g., more highly-substituted congeners) over others. Ongoing work by groups with greater expertise in protein structure determinations, especially the van der Meer group, is attempting to determine a structure for HbpR. However, this class of regulatory proteins has presented much difficulty in expression and purification of isolated, full-length protein. This has hindered the determination of the structure of HbpR.

In chapter four, truncated fragments of the extensively studied glucose binding protein (GBP) from *E. coli* were engineered to function as a biosensor for glucose. Native GBP has been previously engineered such that a fluorophore, 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC), was attached at a unique cysteine at position 152. However, the full-length native protein has been found to have a binding affinity in the micromolar range. In order to be useful in a sensing system for physiologically-relevant glucose concentrations, the binding affinity would need to be in the millimolar range. To that end, we designed truncated glucose recognition peptides (tGRPs) based upon the template of full-length GBP. We hypothesized that by truncating the native protein, we may be able to see a shift in the binding affinity, in addition to other changes in the characteristics of the protein. Indeed, we observed that as the protein was truncated to a greater extent, the binding affinity shifted to higher concentrations of glucose. However, truncating the native protein also demonstrated the negative effect of reducing the thermal stability of the protein, resulting in a melting temperature much lower than full-length GBP. Preliminary work in our laboratory has circumvented this through the global incorporation of

fluorinated unnatural amino acid analogues that are known to increase the thermal stability in other proteins. Additionally, by identifying portions of the protein that must be conserved to maintain a functional protein, we are moving toward a small enough protein that the possibility of constructing the protein using peptide synthesis methods may become a reality. This would allow us greater control over customizing the protein by easily introducing site-specific mutations. Additionally, the library of unnatural amino acids available for incorporation into the amino acid sequence is more extensive when using solid state protein synthesis.

The fifth chapter details the pursuit of another protein-based biosensor for glucose based upon the hyperthermophilic glucose binding protein (tmGBP) from the extremophil, *Thermotoga maritima*. We chose this protein because of its exceptional thermal stability; in the presence of glucose the melting temperature of tmGBP is well over 100 °C. Improved thermal stability should improve sensor lifetime and facilitate a more rugged sensor, which can perform in more extreme conditions and withstand a greater variety of storage and transport conditions. Again, in this work, we sought to develop truncated fragments of the full-length protein in an effort to realize a minimized protein structure that would afford all of the benefits mentioned previously. This work also relied on the intrinsic fluorescence of a single tryptophan residue located within the binding pocket of the protein instead of an exogenous fluorophore. The use of intrinsic protein fluorescence mitigates the need for extra labeling and purification steps.

Future work for each of the protein-based sensing systems will also involve further alteration of the protein structures and, as a consequence, their response properties by incorporation of unnatural amino acid analogues. This work is already ongoing in our laboratory with the incorporation of fluorinated tryptophans and leucines into GBP and the tGRPs discussed in chapter 4. Results thus far suggest that incorporating these fluorinated amino acids, especially the fluorinated leucine, increases the thermal stability of the protein. Other avenues of continued research in this vein include the incorporation of unnatural amino acids which can themselves be used as the reporter thus negating the need for an extra fluorophore labeling step. For example, we are pursuing the incorporation of electroactive amino acids, such as benzoylphenylalanine in an effort to yield a protein which would elicit an electrochemical response upon glucose binding. The palette of unnatural amino acids which can be incorporated during *in vivo* expression is somewhat limited based on the availability of auxotrophic strains and orthogonal tRNA/synthetase pairs. Part of the motivation for truncating the proteins in the development of our sensing systems is the elucidation of minimized protein structures and identification of essential sequences necessary for protein function. The identification of shorter amino acid sequence capable of functioning as a sensor for a desired analyte will allow for the possibility of solid phase peptide synthesis which greatly increases the variety of unnatural amino acids which can be employed.

A final near-term pursuit of these biosensing systems will entail the incorporation into devices. Some work in this direction has been pursued by our laboratory with the incorporation of protein based biosensors for glucose into fiber-optic based devices. Preliminary work has been accomplished that demonstrates the proteins can be incorporated and maintain their binding and sensing activities. Future pursuits should include detailed characterization of these devices with regards to their analytical properties such as sensitivity, selectivity, reproducibility, and lifetime. Also, validation of the response of the device with real samples will need to be performed. In addition, physical characteristics such as device storage, ruggedness, and transport will need to be studied. Finally, the effects and mitigation of an immune response to an implanted fiber optic sensor will need to be investigated.

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