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ENGINEERING PROTEINS WITH UNIQUE CHARACTERISTICS FOR DIAGNOSTICS AND BIOSENSORS

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ABSTRACT OF DISSERTATION

Smita Joel

The Graduate School

University of Kentucky

2011

ENGINEERING PROTEINS WITH UNIQUE CHARACTERISTICS FOR
MOLECULAR DIAGNOSTICS AND BIOSENSORS

ABSTRACT OF 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

Smita Joel

Lexington, Kentucky

Director: Sylvia Daunert, Professor of Chemistry

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ABSTRACT OF DISSERTATION

ENGINEERING PROTEINS WITH UNIQUE CHARACTERISTICS FOR MOLECULAR DIAGNOSTICS AND BIOSENSORS

Proteins possess a broad range of structural and functional properties and, therefore, can be employed in a variety of biomedical applications. While a good number of protein-based biosensing systems and biosensors for target analytes have been developed, the search for versatile, highly sensitive and selective sensors with long term stability able to provide fast detection of target analytes continues to be a challenge. To that end, we now report the design and development of modified proteins with tailored characteristics and their further utilization in the development of biosensing systems.

We take advantage of binding proteins that undergo a change in conformation upon binding to their respective target ligand analytes for the development of highly selective biosensing systems. The first class of binding proteins that was explored for this purpose was antibodies. A non-canonical site in the variable region of a monoclonal antibody was tagged with a fluorescent probe to sense the binding of analyte to its corresponding antigen-binding site. The strategy employed for designing antibody-sensing molecules is universal as it can be employed for sensing any biomolecule of interest provided that there is an available antibody against the target ligand analyte.

In a second strategy, we utilized designer glucose recognition proteins (GRPs) that were prepared by incorporation of unnatural amino acids in the glucose/galactose binding protein (GBP) of *Escherichia coli* and its truncated fragments. By taking advantage of the global incorporation method, we were able to fine-tune the binding affinity and thermal stability of the proteins, thus, allowing for the development of a reagentless fluorescence based fiber optic glucose biosensor capable of monitoring glucose in the hypoglycemic, normal, and hyperglycemic range, as well as in the hypothermic and hyperthermic temperature range.

Keywords: protein-based biosensing systems, antibody, glucose recognition proteins, global incorporation of unnatural amino acids, fiber optic glucose biosensor

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ENGINEERING PROTEINS WITH UNIQUE CHARACTERISTICS FOR MOLECULAR DIAGNOSTICS AND BIOSENSORS

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DISSERTATION

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CHAPTER ONE

INTRODUCTION

Proteins are heteropolymers of amino acids with a wide array of functions and properties. Emerging challenges in medicine, discovery science, and engineering present an ongoing demand for modified proteins with enhanced functionality and properties. For many years scientists have relied on nature for producing proteins that evolved into new ones with different characteristics. A new field of engineering known as protein engineering emerged due to advances in the field of DNA technology in the 1970s. Protein engineering provides the basis for the design and construction of new or improved proteins. Protein engineering has provided new insights into protein structure and function. The principles of gene modification set forth by the capability of preparing synthetic oligonucleotides in the early 1970s,[1, 2] allowed for the first report on site-directed mutagenesis to modify DNA by Smith and co-workers.[3] However, the actual use of site-directed mutagenesis to introduce changes at known sites in structural genes with the aim of studying and modifying protein function was not reported until the early 1980s.[4] Specifically, mutants of tyrosyl-tRNA synthetase and β-lactamase were among the first proteins to be prepared by these methods and analyzed.[5] However, another stumbling block in the field of protein engineering was its inability to modify proteins specifically at predetermined positions as the knowledge of three-dimensional structure was still vague. The advent of new technologies that enabled solving protein structures by X-ray crystallography in a nearly routine manner proved to be crucial in advancing the field of protein engineering. Recent advancements in interactive graphics and computer

modeling softwares along with their use by biological chemists and biotechnologists have stimulated knowledge-based design of proteins, thus providing better understanding of the structure and function of proteins.

Today, scientists can design and create proteins using a plethora of genetic-based, posttranslational, and chemical modification methods. Figure 1 shows the steps involved in designing and creating functional proteins. The first step in designing a new protein involves knowledge of the original protein structure to be modified. This information is provided by the X-ray crystal structure or 2-D NMR of the protein to be modified, which is prepared by expression, purification, and characterization of its functions followed by its crystallization. A thorough study of the original protein structure gives an insight about the locations within the original protein that can be altered to endow the protein with target modifications. The protein is then modified at the genetic level or by chemical means, and subsequently evaluated to assess whether the target characteristics have been incorporated. The cycle continues as the new protein can again be studied and modified using the same methods.

In this dissertation work, various protein engineering methods are employed and discussed. However, the main focus remains the engineering of designer proteins by global incorporation of unnatural amino acids and by chemical modification of immunoglubulins via photolabeling. Specifically, designer proteins for glucose sensing were developed by global incorporation of unnatural amino acids within the Galactose/Glucose Binding Protein (GBP) of *E. coli*. These designer glucose recognition proteins (GRPs) with improved stability and activity were then incorporated into a hydrogel network, which
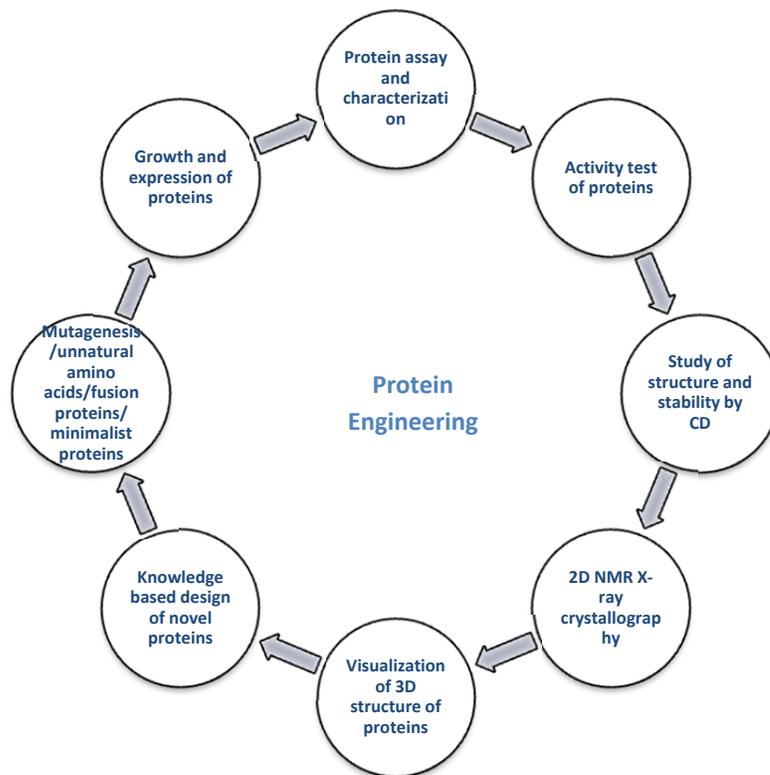
Figure 1.1 Schematic showing the steps involved in the design and engineering of novel proteins. Figure adapted from Blundell, et al, Phil. Trans. R. Soc. Lond. B 324, 448, 1989.

served as the sensing material in fiber optic biosensors for glucose. Designer antibodies were prepared by photolabeling at an unconventional site within the variable region of the antibody, and utilized for developing universal biosensing systems for various biomolecules.

Mutagenesis

The properties and functions of proteins can be altered at the genetic level. The process for generating amino acid coding changes at the DNA level is called mutagenesis. These changes in the amino acids can be generated in a site-directed or random manner. With the availability of X-ray crystallographic structures of proteins, it is possible to determine which amino acids in a protein can be changed to attain a specific property. Mutations can either be point mutations, wherein one amino acid is replaced by another, or deletion mutations in which an amino acid is deleted from the amino acid sequence of a protein, or insertion mutations where an amino acid is inserted in the amino acid sequence. However, if the amino acids to be changed in order to alter the properties or functions of a protein are not known, then random mutagenesis can be carried out. This approach has two advantages. First, detailed information about the function of amino acids in the protein to be altered is not required. Secondly, this approach results in an array of protein mutants with potentially interesting properties. Methods to create random mutants include XL1-Red competent cells, error-prone PCR and degenerate oligonucleotides-Pfu (DOP).[6] XL1-Red cells are engineered *E. coli* with DNA repair deficiency. In this method the plasmid bearing the DNA of interest is transformed into

the cells and mutations occur in each round of replication. In error-prone PCR the polymerase has high error rate. During amplification mutations are introduced by altering the ratio of nucleotides and the concentration of divalent cations.[7] DOP mutagenesis involves the use of two degenerate oligonucleotides as primers. The position of mutation can be controlled by adjusting the ratios and position of degenerate nucleotides during the synthesis of degenerate oligonucleotides.

Point and deletion mutations can lead to the construction of proteins with new properties. Point mutations have been utilized in several proteins to introduce amino acids for covalent attachment of fluorophores. Hence, this protein engineering technique can be used to integrate optical signal transduction functions directly into proteins. This method has been used for developing fluorophore labeled binding protein biosensors for several analytes, including glucose, amino acids, anions, cations and dipeptides.[8] A fluorescent biosensor family was constructed by mutating several periplasmic binding proteins to covalently attach environment sensitive fluorophores to the proteins.[8] Single cysteine mutations were performed at desired locations in the protein, which allowed covalent attachment of environment sensitive fluorophores in or near the ligand binding pocket of the protein. The biosensors can detect the presence of the ligand/analyte, which is measured as a dose-dependent change in the fluorescence of the fluorophore upon ligand binding.[8-10] Besides fluorescent reporter molecules, several other molecules can be covalently attached to mutated proteins for immobilization purposes.

Thermostability of proteins can be improved by site-directed mutagenesis. Point mutations have been engineered in the yeast enzyme triosephosphate isomerase to replace two different asparagines with threonine and isoleucine. Converting the asparagines to

threonine and isoleucine enhanced the thermostability of the enzyme. However, replacing an asparagine with aspartic acid resulted in reduced thermostability of the protein.[11] Thermostability of proteins can also be enhanced by adding disulfide bonds within the structure of the protein, which can be achieved by introducing cysteine residues via site-directed mutagenesis. T4 lysozyme in its native form has two cysteines, neither of which is involved in a disulfide bond. The melting temperature of T4 lysozyme is 41.9 °C. It has been reported that introducing cysteines that are involved in the formation of disulfide bonds enhances the thermal stability of T4 lysozyme. The results demonstrated that the thermal stability increases as the number of disulfide bonds increases, with the most thermostable mutant being the one with the largest number of disulfide bonds (Table 1.1).[12]

The catalytic activity of proteins can also be modified by site-directed mutagenesis. This has been proved by engineering two point mutations in tyrosyl-tRNA synthetase of *Bacillus stearothermophilus*. One mutation, Thr51-Ala51 was done to get rid of the hydroxyl group and the other, Thr51-Pro51 was performed to distort the local polypeptide backbone. Both mutations increased ATP binding activity in the enzyme. The mutant with Pro51 was found to bind ATP 100-fold stronger than the native enzyme.[13]

Furthermore, mutagenesis has been utilized to engineer proteins for therapeutic purposes. For example, insulin has been engineered through site-directed mutagenesis to create rapid acting (lispro, aspart and glulisine) and long acting (glargine and detemir) insulin analogs.[14] The rapid acting insulin analogs were constructed by replacing or switching one or two amino acids. These mutations in insulin reduce the tendency to

Table 1.1 Melting temperature of wild type and engineered mutants of T4 lysozyme. Adapted from Matsumura, et al, Nature 342, 291-293, 1989.

Enzyme	No. of disulfide bonds	T_m (°C)
Wild type	0	41.9
3C-54T	1	46.7
D3-97/9-164	2	57.6
D9-164/21-142	2	58.9
T3-97/9-164/21-142	3	65.5

form hexamers, thus facilitating rapid absorption. The long acting insulin analogs were obtained by substitution or deletion of specific amino acids. These changes resulted in a shift in the isoelectric point of insulin from 5.4 to 6.7, which prolongs its dissociation at physiological pH conditions. Another strategy to engineer proteins for therapeutic purposes is the development of catalytic antibodies. Antibodies are proteins that are used by the immune system to identify and neutralize foreign agents. However, mutagenesis has enabled the development of catalytic antibodies that function as enzymes and catalyze reactions. Some of the potential therapeutic applications include detoxification after accidental exposure to insecticides,[15] prevention of cocaine addiction and overdose,[16, 17] and prodrug activation in cancer treatment.[18]

Fusion Proteins

Partial or full protein sequences can be joined together via recombinant DNA technology resulting in fusion or hybrid proteins. These proteins can have partial or full functions or characteristics of the constituent proteins. Fusion proteins have extensively been used to study interactions between proteins. Additionally, a binding protein can be fused to an inherently fluorescent protein, such as the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, to develop protein-based sensing systems. Upon binding to the analyte, the target binding protein undergoes a change in the conformation, which can be detected as a dose-dependent change in the fluorescence of the reporter protein, GFP. Furthermore, fluorescent fusion proteins have been used to study the location, movement and degradation of proteins in living cells.[19] Another application of

fusion proteins is to aid in the purification of proteins via affinity chromatography. A short sequence of amino acids, such as histidines, are fused to either the N- or C-terminus of a protein allowing for its purification by nickel affinity chromatography; for example, in this dissertation work a six histidine sequence was inserted at the C-terminus of the gene encoding the glucose binding protein for purification via nickel affinity. Small peptides[20] or antibody fragments[21] can also be fused to proteins for purification purposes. For example, fusion of antibody fragments to proteins enables purification via interactions with protein A. Fusion protein technology has been employed to develop biosensing systems for peptides,[20] drugs, zinc and heme, among others. An octapeptide was fused to GFP in order to develop a fluorescence binding assay for the peptide. The immunoassay was based on the sequential binding of the free octapeptide and GFP labeled octapeptide to anti-octapeptide antibodies on a solid surface.[22] A zinc sensor was developed by fusing histidine tags at each end of a fusion protein comprised of the enhanced cyan and enhanced yellow fluorescent proteins joined together by a linker. Since the hexahistidine tags undergoe dimerization in the presence of zinc, fluorescence resonance energy transfer occurs between the two fluorescent protein domains, thus resulting in the ratiometric detection of zinc in nM ranges.[23] In another example, cytochrome b_{562}, a heme-binding protein, was fused to the N-terminus of the enhanced green fluorescent protein (EGFP) to create a heme sensor. The detection of hemin was based on the fluorescence quenching due to energy transfer between EGFP and cytochrome b_{562} in the presence of hemin.[24]

Split protein technology, which is employed to study protein-protein interactions[25, 26] and protein-nucleotide interactions, is a step further in the field of protein

engineering.[27] The split protein methodology includes one and two component systems. A one component system consists of two fragments of a reporter protein attached to two peptides or nucleotides. The two component system has been exemplified by fragmenting GFP and attaching the two inactive fragments to two leucine zippers. The leucine zippers come together via non-covalent interactions, bringing the two fragments of GFP together, hence producing fluorescence.[28] When two fragments of a reporter protein are attached to the two termini of a binding protein in a one component system, the binding of the ligand to the binding protein results in a conformation change that brings the two fragments of the reporter protein closer, hence producing a signal. This approach can be utilized to develop biosensing systems for various biomolecules by inserting the sequence of a biomolecule binding protein within the sequence of a reporter protein. Conformational changes in maltose binding protein (MBP) have been studied using the one component split methodology. Two fragments of GFP were attached to the N- and C-terminus of MBP. Upon binding to maltose, the two fragments of GFP were brought together and resulted in changes in fluorescence. The changes in fluorescence in response to maltose were detected in a concentration and time dependent manner.[29] This sensing strategy is also referred to as molecular switches,[30, 31] and has been utilized for inserting MBP,[29] GBP[32] (Figure 1.2), and calmodulin[33] within split reporter proteins such as GFP, aequorin, and EGFP, respectively. The split protein methodology allows for two functionally and structurally unrelated proteins to be integrated, thus combining their characteristics; hence, it can prove to be an important tool for designing molecular sensors for analytical and therapeutic applications.[34]
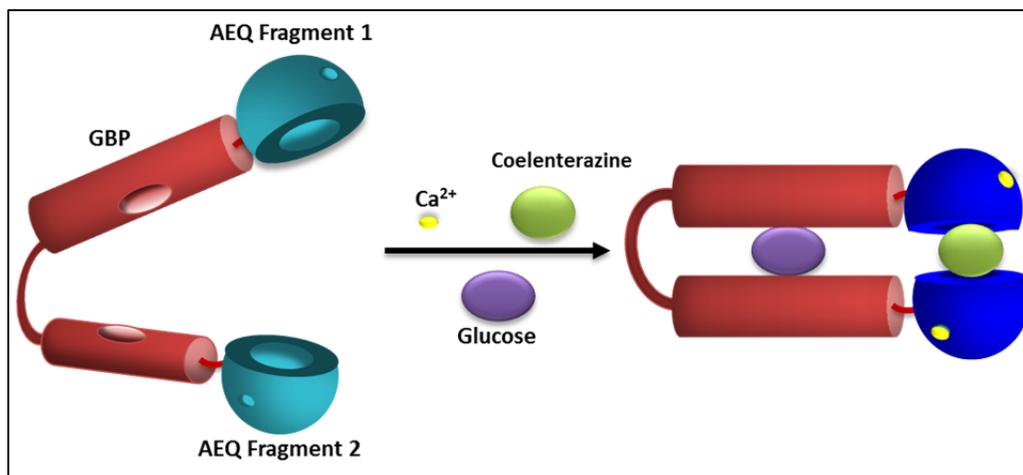
Figure 1.2 Schematic of a protein switch showing the components (GBP, AEQ fragments) and triggers (glucose, calcium ions, coelenterazine) that result in switching from the "off" to "on" mode.

Minimalist Proteins

Proteins are macromolecules comprised of hundreds of amino acids. However, only a fraction of the protein structure or amino acid sequence in a protein is required for maintaining a specific biological activity. Hence, efforts have been made to create a minimum portion of a protein structure required to retain the desired function. For example, in an enzyme or a binding protein the substrate or ligand binds to an active site consisting of a specific sequence of amino acids; however, the amino acid sequence of the rest of the protein helps in maintaining the required structure of the active site.[35] The truncation of proteins to generate protein fragments that contain the amino acid sequence essential for maintaining the protein biological function has been explored in our lab. GBP is one example of such proteins subjected to truncation.

The galactose/glucose binding protein (GBP) from *E. coli* is a periplasmic protein involved in bacterial chemotaxis and active transport of glucose and galactose.[36] GBP is a monomer with a molecular weight of 33 kDa, consists of 309 amino acids, and binds to glucose and galactose present in μM ranges.[37] GBP is ellipsoidal in shape and consists of two globular domains held together by three peptide strands referred to as the hinge region.[10] The two domains have a core of six strand β-sheet flanked on both sides by two or three helices.[38] In the open form (Figure 1.3), when glucose is not bound, the two domains are far apart and the ligand binding site is deep within the cleft and accessible to solvent. On the other hand, in the closed form (Figure 1.3) glucose is bound in the cleft between the two domains and the ligand binding site is solvent-inaccessible, which results in the formation of an extensive network of hydrogen bonds between the polar residues of the binding site and the sugar.[39] The binding of glucose is accompanied by a
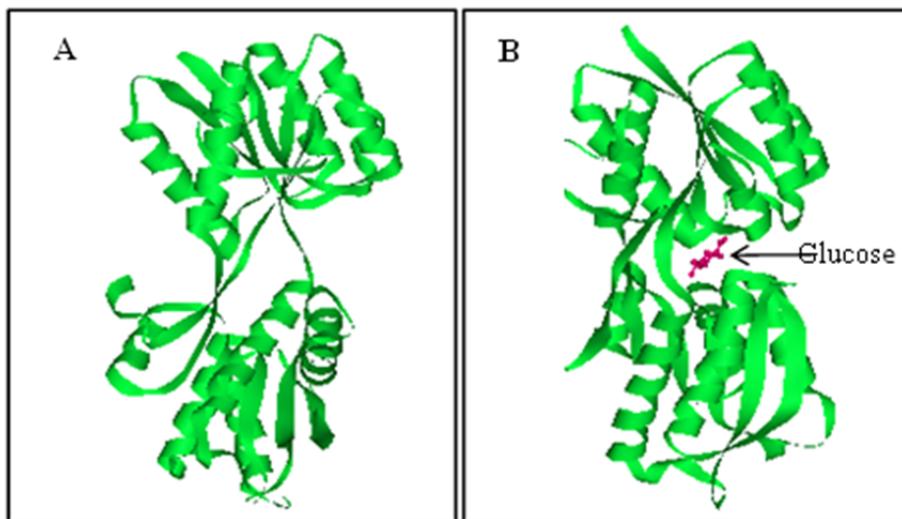
Figure 1.3 Crystal structure of GBP without glucose (A, open form) and with glucose (B, closed form).

conformational change of the protein at the hinge region. The bound sugar is stabilized within the binding pocket by a series of interactions, including hydrogen bonds, van der Waals interactions and salt bridges. Upon binding to the sugar, 13 strong hydrogen bonds are formed between the sugar and the protein, involving eight polar side chains of GBP distributed between the two domains and a water molecule. Additionally, the glucopyranose ring is stacked on both sides with aromatic residues. The presence of glucose bound to GBP has been found to increase the stability of the protein structure and restore the cooperativity of temperature-induced transitions.[40] In addition to the sugar binding site, GBP also has a calcium binding site in the C-terminal domain. The calcium binding site is constituted by a nine residue (134-142) loop.[41]

GBP was truncated at both the N- and the C-terminus generating protein fragments. Three fragments of GBP were thus obtained. The largest one had amino acids 14 through 296, the second fragment had 14 through 256, and the smallest one had 87 through 271. The smallest fragment did not bind to glucose; hence, it lost its activity upon truncation, suggesting that the amino acids removed from the protein sequence were essential for glucose binding. However, the two larger fragments of the full length GBP (Figure 1.4) showed glucose binding activity.[42] This observation suggested that the full length amino acid sequence is not necessary for maintaining the activity of GBP. Therefore, utilization of the minimalist strategy allows for the creation of smaller functional proteins. The minimalist protein strategy can also be utilized to study folding of the protein and its active site.[43]
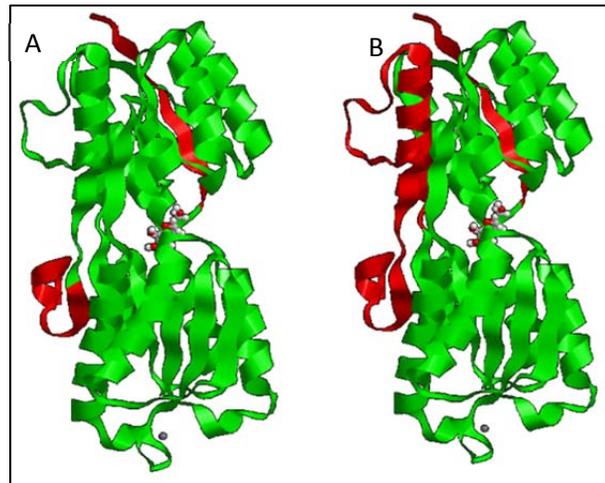
Figure 1.4 Crystal structure of GBP with glucose. The red portions in the structure indicate the parts of the amino acid sequence that have been eliminated. A: GBP fragment with amino acids 14 through 296, B: GBP fragment with amino acids 14 through 256.[42]

Incorporation of unnatural amino acids

A key advancement in the field of protein-based biosensors is the design of proteins with improved binding selectivity, specificity and affinity for their ligands, with enhanced stability of the protein. Protein engineering methods are generally based on chemical and genetic methods. A more recent, less studied method of engineering proteins involves the incorporation of unnatural amino acids in place of natural amino acids in proteins, either

at a particular site (site-specific) or throughout the protein (global). This is a challenging method as it plays with the natural fidelity and proof reading mechanism that living organisms are endowed with. Nature has provided 20 natural amino acids that are vital for the existence of organisms. Replacing the natural amino acids with unnatural amino acids might result in the production of undesired or incorrect proteins, which can lead to cell death. However, emerging techniques and new methods have allowed expanding the genetic code beyond the one nature has provided.

Expanding the genetic code via incorporation of unnatural amino acids allows exploring the structure and function of proteins by manipulating the amino acid backbone. Also, this method allows for the tailoring or tuning of properties and functions of proteins. For example, if leucine in a protein is replaced by an unnatural analogue of leucine, such as fluoroleucine, then the change in the structure of the protein can be studied. Fluorine and hydrogen have similar van der Waals radii; therefore, hydrogen can easily be replaced with fluorine in amino acids, producing minimal steric perturbation in proteins. However, high electronegative and hydrophobic characteristics endow proteins

with distinct properties. Several fluorinated amino acids, such as, fluorovaline, fluoroleucine, fluoroisoleucine, fluorophenylalanine, and fluoroproline have successfully been incorporated into the structure of proteins.[44] Besides providing an insight into the structure of a protein, incorporation of unnatural amino acids allows for introducing chemical scission sites and sites for photoaffinity labeling. It also enables site-specific conjugation and immobilization. A variety of methods for the incorporation of unnatural amino acids in proteins have been developed, which include solid phase peptide synthesis, semi-synthesis, *in vitro* site-specific incorporation, *in vivo* site-specific incorporation, and global incorporation. All of these methods will be discussed in the following sections. In our efforts towards improving the activity and stability of GBP we utilized the global incorporation of unnatural amino acids into the structure of GBP.

Solid phase peptide synthesis

One of the most used methods for incorporation of unnatural amino acids is solid phase peptide synthesis. This is a synthetic method for producing peptides or proteins that are difficult to express in bacteria or to introduce unnatural amino acids in peptides. This method employs the carboxyl group of one amino acid for coupling with the amino group of another amino acid, while protecting the amino or carboxyl group of amino acids not involved in peptide bond formation. In peptide synthesis an amino acid is bound to a resin via the carboxyl group and the amino group is protected. The amino group is then deprotected and reacted with the carboxyl group of another amino acid having the amino

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group protected. This method involves repetitive cycles of deprotecting, coupling and washing. The synthesized peptide remains covalently attached to the solid support until cleaved by anhydrous hydrogen fluoride or trifluoroacetic acid. Two important protecting groups used for protecting the α-amino group are Fmoc (9-fluorenylmethyloxycarbonyl)[45, 46] and *t*-Boc (tert-butoxycarbonyl).[47, 48] A variety of solid supports, such as, polystyrene or polyacrylamide resins can be used. This method is well standardized and automated; however, it has a drawback, that is, only small peptides comprising up to 100 amino acids can be synthesized. This chemical method can also be used for incorporating unnatural amino acids in peptides. However, the peptide produced may not fold properly or show the desired activity. The desired activity of proteins may be achieved by post-translational modification of the protein within the cell. However, the desired activity of protein cannot be obtained using the chemical synthesis of peptides. The demand for bigger peptides or proteins still remains; to that end, semi-synthesis of proteins can be performed.

Semi-synthesis of proteins involves chemical ligation of either two naturally existing smaller peptides or two peptides synthesized via solid phase peptide synthesis or one natural and one synthesized peptide. A major advancement in this field was the chemoselective linkage[49] of a peptide containing an amino-terminal cysteine with a peptide containing a carboxy-terminal thioester, attained by the initial transthioesterification followed by S,N-shift to produce a native amide bond. Hence, this method can be used for generating recombinant proteins. Amino-terminal cysteines can be introduced in proteins using proteases,[50] while a thioester can be introduced into a recombinant protein by fusion to an intein. The peptide with carboxy-terminal cysteine

then reacts with the protein thioester by native chemical ligation, resulting in a semi-synthetic protein. This method is known as expressed protein ligation (EPL).[51, 52] This method allows the generation of bigger peptides/proteins and somewhat overcomes the limitations posed by post-translational modification of proteins. A drawback of this semi-synthesis method is that it requires the introduction of an N-terminal cysteine or the presence of cysteine within the structure of the protein, which in some cases may perturb the structure or activity of the protein. The incorporation of unnatural amino acids at desired locations within proteins is also limited when using EPL methods as the unnatural amino acid can only be introduced in the synthetic part of the peptide. Thus, the need to incorporate unnatural amino acids without any location restrictions has been achieved by exploiting the natural biological machinery that the cells use for manufacturing proteins.

Site-specific incorporation of unnatural amino acids

The fidelity of the genetic code depends upon the interaction of codon-anticodon and the acylation of tRNA with the proper amino acid by aminoacyl-tRNA synthetases (aaRSs). Thus, changing the genetic code requires a tRNA for a specific codon and a method of acylating the tRNA with an unnatural amino acid. Nature has provided 64 three base codons in the standard genetic code. Three of these three base codons, namely, UAG (amber), UAA (ochre) and UGA (opal) are referred to as stop codons or nonsense codons as they signal the termination of translation. The amber codon is the least used stop codon in *E. coli*, and a number of efficient 'suppressor' tRNAs are known.[53] The

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suppressor tRNAs are tRNAs from certain organisms that have the capability to 'read through' a stop codon. Additionally, the presence of the amber suppressors in some *E. coli* strains does not significantly affect cell growth rates.[54]

Schultz and Chamberlin have reported *in vitro* incorporation of unnatural amino acids by "nonsense suppression" strategy. Nonsense suppression involves the use of nonsense codons and suppressor tRNAs. The first step towards incorporation of unnatural amino acids via this method is the construction of aminoacylated suppressor tRNA. Hecht and coworkers have constructed a suppressor tRNA by the condensation of the 3' end of an *E. coli* truncated tRNA and an acylated dinucleotide in the presence of T4 RNA ligase, hence resulting in an aminoacyl-tRNA (aatRNA) charged with an unnatural amino acid.[55, 56] This methodology has been further improved by Schultz[57] and Sisido.[58]

Dougherty and coworkers have been successful in incorporating unnatural amino acids into proteins expressed in Xenopus oocytes via the nonsense suppression method. The oocyte is coinjected with the modified mRNA encoding for the target protein and the aatRNA is chemically acylated with an unnatural amino acid. This results in the expression of the protein containing the unnatural amino acid on the surface of the oocyte. This methodology has enabled a detailed study of the structure-function relationships of ion channels and the incorporation of biophysical probes.[59]

Further, Schultz and coworkers have developed an alternative to the nonsense supression method for incorporating unnatural amino acids into proteins *in vivo*. This method (Figure 1.5) utilizes a novel tRNA/aaRS pair for each unnatural amino acid to be incorporated. The aaRS is engineered such that it can only recognize the unnatural amino acid and proficiently acylate the tRNA. The tRNA/aaRS pair should be orthogonal to the
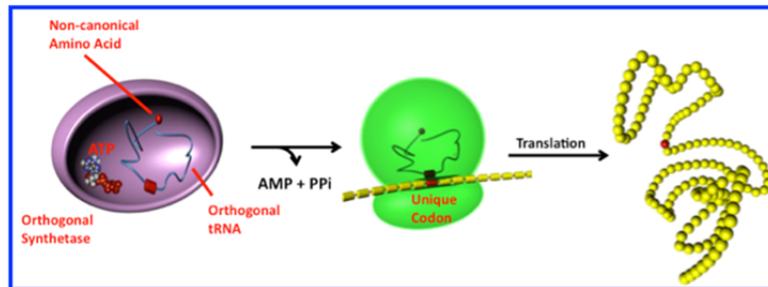
Figure 1.5 Site-specific unnatural amino acid incorporation method. The specific site on the protein's gene is first mutated to TAG. The genes encoding for the orthogonal tRNA/aminoacyl-tRNA synthetases are cotransformed into *E. coli* with the genes for the TAG-mutated protein.

host organism, meaning that the aaRS must not recognize endogenous tRNAs and the suppressor tRNA cannot be a substrate for endogenous aaRSs. The most commonly used tRNA/aaRS pair has originated from *Methanococcus jannaschii*. The tRNA/aaRS pair from *M. jannaschii* has been used to incorporate various unnatural amino acids, such as photoactivatable amino acids[60] and keto-containing amino acids,[61] into proteins. Several other orthogonal tRNA/aaRS pairs have been proposed to suppress nonsense codons in eukaryotic cells, including mammalian cell lines.[62] Schultz and coworkers have efficiently incorporated five amino acids with high fidelity in the genetic code of *Saccharomyces cerevisiae* by utilizing a unique orthogonal tRNA/aaRS pair originated from *E. coli*.[63]

Site-specific incorporation of unnatural amino acids allows for the precise positioning of the desired unnatural amino acid. However, the nonsense suppression approach for incorporation of unnatural amino acids suffers from the limitation that a maximum of two unnatural amino acids can be incorporated in a protein at a time, due to the availability of two nonsense codons. Sisido and coworkers have overcome this limitation by using extended codons and frame shift suppression. However, if these extended codons are read as a three base codon by an endogenous tRNA, the reading frame will be shifted by one base. This will eventually result in a premature encounter with a stop codon and early termination of protein synthesis, thereby resulting in a truncated protein. Several four and five base codons have been used to incorporate unnatural amino acids in proteins in *E.coli*.[64-67]

The in *vivo* method has been used to incorporate a wide variety of unnatural amino acids in proteins. These include chemically reactive amino acids containing

functional groups, such as, ketones and photoreactive groups capable of covalent bonding, activation via photocages, and photoisomerization upon irradiation with UV light. Fluorescent amino acids have also been incorporated. An amino acid analogue of prodan, an environment-sensitive fluorophore, has been successfully incorporated in yeast proteins.[68] Incorporation of fluorinated amino acids in proteins causes minimal perturbation of the protein structure because the van der Waals radii of fluorine and hydrogen are similar. Incorporation of trifluorleucine in coiled-coil proteins has shown to enhance stability. The 4-iodo-l-phenylalanine residue can be used for the chemoselective modification of proteins. Rowe et al. have recently incorporated four unnatural phenylalanines (bromo, iodo, amino and methoxy derivatives) in place of tyrosine at position 82 in aequorin. Aequorin is a bioluminescent protein with an emission wavelength at 473 nm. The incorporation of unnatural phenylalanine resulted in a spectral shift of up to 44 nm in aequorin.[69]

Global incorporation of unnatural amino acids

Site-specific incorporation of unnatural amino acids enables the study of protein structure and function. It also allows the introduction of new functions in the protein by adding novel functional groups. However, the number of unnatural amino acids that can be introduced in a protein via site-specific incorporation is very limited. The global incorporation method allows the incorporation of unnatural amino acids throughout the protein; hence, it helps in better understanding the structural and functional properties of a protein. Global incorporation of β-selenolo[3,2-b]pyrrolyl-L-alanine in the small

protein barstar allows for solving phase problems in protein X-ray crystallography.[70] Global incorporation involves the replacement of a single natural amino acid at all positions within the protein by its unnnatural amino acid analogue. For example, if an unnatural derivative of lysine is incorporated in a protein via the global method, then all the lysines in the protein are theoretically replaced by the unnatural analogue. This method requires that the unnatural analogue is structurally very similar to the natural amino acid, so that it is readily accepted by the natural tRNA in the genetic/synthetic machinery of the organism. An unnatural amino acid that differs considerably from the natural amino acid might lead to no or partial incorporation in the protein. Another requirement is the use of an auxotrophic host strain. An auxotrophic strain is a cell strain that cannot grow in the absence of a particular amino acid. Therefore the growth of such strains requires the addition of that particular amino acid in the growth medium. This method utilizes the natural machinery of the cells for protein synthesis and does not require special tRNAs or ribosomes. To incorporate an unnatural amino acid globally, the auxotrophic strain for the amino acid that will be replaced by its unnatural analogue is grown in a growth medium enriched with all 20 amino acids. The cells are then harvested by centrifugation and then washed thoroughly to get rid of the growth medium. The cells are then grown again in a medium that is enriched with all amino acids except for the one that needs to be replaced by its unnatural analogue, followed by the addition of the unnatural analogue. This method of global incorporation is known as the "medium shift method." The possibility of tuning the properties of proteins via global incorporation was proven by incorporating norleucine in place of methionines in a cytochrome, which increased the peroxygenase activity by two-fold[70]. The hydrophobicity and spectral

properties of barstar were tuned by replacing natural tryptophans with aminotryptophans.[71] In another example 5-hydroxytryptophan and 7-azatryptophan were incorporated in staphylococcal nuclease. An incorporation efficiency of 98% was reported. The secondary structure was found to be unaltered by the incorporation, but the stability of the proteindecreased upon incorporation of unnatural analogues.[72] Bae and coworkers incorporated unnatural tryptophans in the green fluorescent protein (GFP), thus shifting the emission spectra and achieving a "gold" variant of GFP.[73] In another example the global method was used to incorporate six unnatural amino acids, p-amino-L-phenylalanine, 3-amino-L-tyrosine, 5-hydroxy-L-tryptophan, 3-fluoro-L-tyrosine, 5-fluoro-L-tryptophan, and 6-fluoro-L-tryptophan, into the structure of aequorin to study their effects on the bioluminescence properties of the photoprotein. A 54 nm shift in the emission maximum of aequorin, when compared to the native aequorin, with a variety of coelenterazine analogues was observed.[74] Thus, global incorporation of unnatural amino acids, such as tryptophan analogues, might facilitate altering the structure of proteins and, consequently, their properties.

Binding Proteins

Binding proteins bind to a specific ligand or class of ligands. These proteins are ubiquitous and, within the cell, the binding of the ligand by the proteins is generally accompanied by the transport of the bound ligand in or out of the cell. For example, GBP is involved in the active transport of glucose across the cell membrane from the

environment into the cell.[75] Binding proteins have been classified on the basis of the ligands they bind. Examples include glucose binding proteins (e.g., GBP, as mentioned previously), calcium binding proteins (e.g., calmodulin, described in a later section), nucleotide (DNA/RNA) binding proteins, and sulfate binding proteins. Binding proteins are also classified on the basis of the mechanism utilized for binding the ligand. An interesting class of binding proteins is that of the so-called hinge-motion binding proteins. Hinge-motion binding proteins undergo a change in the conformation, consisting of the bending of two protein domains around a hinge region, upon binding to their ligands.[76] Many hinge-motion binding proteins are found in the periplasm of bacterial cells and are involved in the transport of molecules and ions across the cell membrane.[77] These periplasmic binding proteins consist of two globular domains that are connected by a hinge region. The binding site is found at the interface of the two domains.[78] The ligand is bound to various residues in the binding site by hydrogen bonds, van der Waals interactions,[79, 80] π-interactions[81] or ionic interactions.[82] Upon binding to the ligand the periplasmic proteins adopt a ligand-bound (closed) conformation and, in the absence of the ligand, the protein adopts a ligand-free (open) conformation. These two forms interconvert through a large bending motion around the hinge. An example of such hinge-motion binding proteins is GBP, which is described in a previous section. Protein engineers and bioanalytical chemists have harnessed the change in binding protein conformation upon ligand binding to develop biosensing systems for several analytes, as previously reported under the section "Mutagenesis". Another class of binding proteins includes antibodies or immunoglobulins, which bind to a wide range of molecules, such as, proteins, nucleotides, drugs, pathogens, other antibodies, etc.

Antibodies as binding proteins

Antibodies or immunoglobulins are glycoproteins that are produced by plasma cells in response to an immunogen or antigen. Antibodies are found in the blood and other body fluids of vertebrates. The basic structure of antibody consists of two heavy chains (50-70 kDa) and two light chains (~30 kDa) linked by disulfide bonds (Figure 1.6). Both heavy and light chains comprise constant and variable regions. Each heavy chain consists of one variable (V_H) and three or four constant regions (C_{H1}, C_{H2}, C_{H3}, C_{H4}) depending upon the antibody isoytype. C_{H1} and C_{H2} are held together by a hinge region which allows flexibility between the two arms of a "Y" shaped antibody. Carbohydrates are attached to the C_{H2} region in most antibodies; however, in some cases they may also be attached at different locations. The antigen binding site resides between the variable regions of the light and heavy chains. The variable region is further divided into hypervariable regions (HV) and framework regions (FR). Most of the variability in the amino acid sequence of the variable region resides in the HV regions. HV regions are also called complementarity determining regions (CDRs). The HV regions form a direct contact with the antigen's surface. The FR regions form a β-sheet structure which serves as a scaffold to hold the HV regions in position to make contact with the antigen. Antibody fragments produced by proteolytic digestion have been used for elucidating structure/function relationships in antibodies. Upon digestion with papain, the immunoglobulin molecule is cleaved at the hinge region generating two Fab fragments, each consisting of the light chain and the V_H and C_{H1} domains of the heavy chain, and one Fc fragment consisting of the remaining constant domains of the heavy chain. Fab is involved in antigen binding.
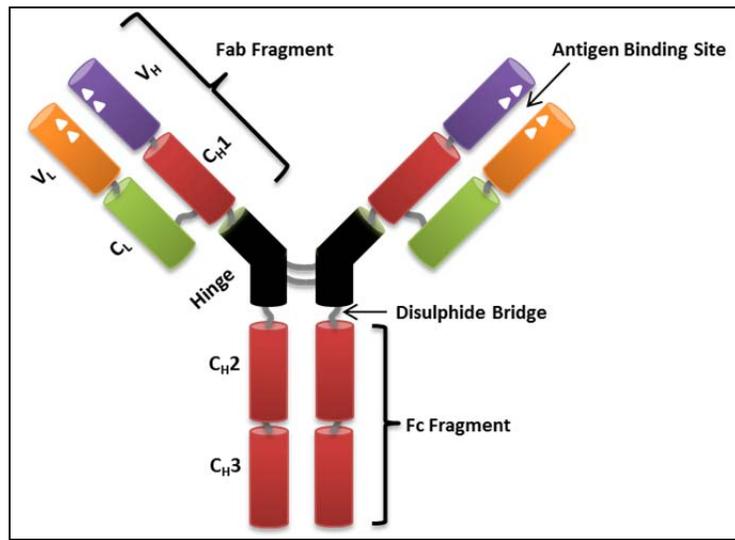
Figure 1.6 Structure of antibody

Fc (so named because it can be crystallized easily) carries out the effector functions. The digestion with pepsin leads to the formation of F(ab')$_2$, containing the two antigen binding sites, and to the cleaving of the Fc fragment into smaller peptides. Immunoglobulins can be divided into five classes based on the differences between the amino acid sequences in the constant regions of the heavy chains. These classes are IgG, IgA, IgM, IgD and IgE. Further, the light chains can be classified as κ or λ chains, based on the amino acid sequence in the constant region of the light chains. IgG are monomers and are the most versatile immunoglobulins, capable of performing all the functions of an immunoglobulin molecule. IgG are the major antibody in serum and the only antibody that crosses the placenta. IgM mostly exist as pentamers. They are the first class of antibody to appear in the serum after exposure to an antigen and the first to be produced in the fetus. IgA are found in external secretions such as saliva, tears, bronchial mucus and intestinal mucus. IgD and IgE exist as monomers. IgE bind to basophils and are involved in allergic reactions.[83]

Antibodies are divided into two types based on their specificity to antigens. Monoclonal antibodies recognize a single, specific epitope of an antigen, while polyclonal antibodies recognize various sites on an antigen. Monoclonal antibodies are usually produced by immunizing a mouse or a rabbit against the antigen of interest to stimulate the production of antibodies. The antibody forming cells are then isolated from the mouse's spleen. These cells are then fused with mouse myeloma cells; the resulting cells are called hybridomas. Each hybridoma produces large quantities of identical antibody molecules. Polyclonal antibodies are produced by immunization of an animal, such as mouse, rabbit or goat. When the animal is injected with the antigen, the B-

lymphocytes produce the polyclonal antibodies, which are collected from the animal blood serum.

Antibodies have been used for several years for the detection of their corresponding antigens. Antibodies labeled with reporter groups form the basis of immunoassays. Immunoassay is an analytical technique for quantitative measurements of target compounds, which found numerous applications in clinical and biomedical diagnostics as well as environmental analysis. The ability of antibodies to form complexes with the corresponding antigens in a highly specific manner results in the great specificifty/selectivity of immunoassays. The most common type of immunoassay in clinical analysis is the enzyme linked immunosorbent assay (ELISA). ELISA can be used to detect specific antigens or antibodies present in the sample. In sandwich-type ELISA, a solid surface (such as polystyrene) is coated with a solution of antibody against the target antigen (capture antibody), and the sample to be analyzed is then added. After incubation, the bound antigen is either detected by the addition of an enzyme-labeled detection antibody specific for the captured antigen (Direct Sandwich) or by first adding the antigen-specific antibody followed by addition of an enzyme-labeled secondary antibody (Indirect Sandwich). The final step is the addition of a chromogenic enzyme substrate, which produces a color whose intensity is determined by absorbance measurements using a spectrophotometer. The label enzyme is usually conjugated to the antibody via chemical coupling, which leads to random attachment of the enzyme to the reactive amino acid side chains of the antibody. This nonspecific coupling of the enzyme to the antibody can lead to various degrees of antibody denaturation or loss of antibody activity or binding ability. To overcome this drawback a method that allows more

specific attachment of enzymes or other molecules to antibodies is required.

Kohler and co-workers have reported the presence of a novel site in the variable antibody domain involving invariant residues, which binds purine containing nucleotide photoaffinity probes with high affinity.[84] This novel site can be utilized for site-specific labeling of antibodies. The nucleotide/nucleoside affinity site is formed by parts of both the light and heavy chains within the variable domain of the immunoglobulin. A computer model of the antibody with adenosine diphosphate (ADP) in the unconventional site was constructed by Kohler and his co-workers (figure 1.7).[84] The model shades light on the biochemical aspects of the interaction between the purine and the site in the Ig molecule.[84] Figure 1.7 also shows the structure and location of the nucleotide with respect to the antigen binding site.[84] The antigen binding site is unaffected by the binding of the ADP molecule in the hydrophobic pocket that forms this unconventional site and that is located below the antigen binding site.[84, 85] The purine ring is held in the hydrophobic pocket via stacking, while the phosphate groups and the ribose are exposed to the solvent between the variable domains of the heavy and light chains of the Ig.[84, 85] The phosphate groups can thus be tethered to molecules like biotin, metal chelates, antisense oligonucleotides, and peptides without affecting the Ig antigen binding site. The purine ring is held between the rings of Trp 103 (in the Ig heavy chain) and Pro 44 (in the Ig light chain) by nonspecific stacking interactions.[84, 85] The fact that these heterocyclic amino acids are highly conserved in most antibodies, and the successful photolabeling of various antibodies suggest that most antibodies can be labeled at this unconventional site.[84, 85] Upon photolysis a covalent bond can be formed between the azido group of the purine ring and the hydroxyl group of Tyr 36 in the light chain as they

Figure 1.7 Computer generated model of the Fv region of the Ig with the inserted ADP.
A: The loops facing the top are the CDRs with the antigen binding site. The red and green
represent the light chain and heavy chain, respectively. B: Interaction of ADP with the
residues Trp-H103, Tyr-L36, and Asp-H101 in the heavy and light chains of the Ig
molecule.[84]

are in close proximity.[84, 85] A similar interaction of the purine ring of the nucleotide probe with the residues Asp 101 or Glu 100 in the heavy chain has been observed in other antibodies.[84, 85]Immunoprecipitation experiments have shown that the average number of probes attached to each antibody molecule is approximately two, and that each of the two arms of the antibody have been labeled with the probe.[84] It has been demonstrated that a biotin molecule can be attached to ATP via the ribose ring or the γ phosphate.[85, 86] These biotin conjugates can then bind to the unconventional site of an antibody in a similar manner as the binding of ADP to the unconventional site.[85, 86] There is no steric hindrance to the antigen binding site of the antibody by this interaction with the biotinylated ATP. This photobiotinylation is performed under mild, physiological conditions, and is quick due to the high affinity of the unconventional site for the nucleotide/nucleoside probes.[85, 86] Since this process involves photoactivated chemical crosslinking, it can be controlled by time and intensity of UV exposure. Also, no residual chemically reactive groups are obtained, as a result of photochemical crosslinking, which would need to be removed.[86] Therefore, this conserved unconventional site of antibodies could be utilized to incorporate signal transduction for the development of biosensing systems. These biosensing systems would be advantageous over ELISA in that they would be ready to use and would require no washing steps. Another advantage of the novel unconventional binding site is its utilization to tether molecules of biological and medical interest to antibodies via coupling to the phosphate groups or the ribose in the nucleotide moiety. We have utilized this novel binding site of antibody to develop a universal biosensing system for biomolecules by covalently attaching a nucleotide fluorescent analog to the nucleotide binding site.

Incorporation of engineered proteins in hydrogels

Hydrogels are three dimensional covalently crosslinked, hydrophilic, water insoluble polymers that swell upon absorbing large amounts of water. Hydrogels have a wide variety of functions related to their swelling, mechanical, permeation, surface and optical properties. These properties make hydrogels suitable for an array of potential applications in the fields of medicine,[87, 88] agriculture[89] and biotechnology.[90] In addition, hydrogels can undergo a change in response to an environmental cue or to a change in environmental conditions. Such stimuli sensitive hydrogels are referred to as "smart" or responsive hydrogels. Various stimuli-responsive hydrogels that respond to pH,[91, 92] temperature,[93, 94] ion concentration,[95] electric potential,[96] and solvent composition[97] have been studied. Stimuli-responsive hydrogels have been utilized in molecular switches, sensors, drug delivery devices, specialized separation systems and artificial muscles.

"Smart" or responsive hydrogels have been made employing protein domains and polypeptides that fold into three dimensional structures with distinct functions. Protein engineering allows for the manipulation of DNA sequences encoding protein domains or peptides and, therefore, allows control over the structure and function of these peptides or domains. Incorporating such engineered proteins within hydrogels can enable manipulation of the properties of the hydrogel. An example of incorporation of such a genetically engineered protein into stimuli-responsive hydrogels has been demonstrated in our lab.[98] In this system, calmodulin, a calcium binding protein, was selected as the biological recognition element in the smart hydrogel. Calmodulin undergoes a large conformational change upon binding calcium, certain peptides and phenothiazines. Calmodulin undergoes two conformational changes: in the presence of calcium it

assumes a "dumbbell" conformation, and in the presence of phenothiazines it goes from the "dumbbell" shape to a more constrictive conformation. A unique cysteine was introduced at the C-terminus of calmodulin by site-directed mutagenesis. This was done to attach an allylamine moiety to the free sulfhydryl residue in the protein. Allylamine allows oriented immobilization in hydrogels. Further, an amine derivative of phenothiazine was reacted with N-succinimidylacrylate to form a polymerizable phenothiazine. The hydrogel was then synthesized by free radical polymerization of allylamine-calmodulin, polymerizable phenothiazine, acrylamide and $N,N^{'}$-methylenebisacrylamide. It was observed that in the absence of Ca^{2+} the hydrogel swelled due to the release of immobilized phenothiazine from the calmodulin binding site and to the change in the conformation of calmodulin, which modifies the hydrophobic surface of the protein, thus altering the water uptake of the hydrogel (figure 1.8). However, in the presence of Ca^{2+}, the phenothiazine was bound to the protein as the phenothiazine binding site was accessible. This creates non-covalent crosslinking in the polymer network, shrinking the hydrogel to its original size.

Stimuli-responsive hydrogels find extensive applications in development of drug delivery systems. An example of such systems is that of glucose sensitive hydrogels for insulin delivery systems. Ishihara et al. developed a glucose responsive hydrogel to control the release of insulin in response to glucose variations.[99] This system consisted of a copolymer membrane of N,N-diethylaminoethyl methacrylate and 2-hydroxypropyl methacrylate combined with a cross-linked polyacrylamide membrane. Glucose oxidase was immobilized within the hydrogel. Glucose oxidase is an enzyme that converts glucose to gluconic acid. In this system glucose diffuses in the membranes and is
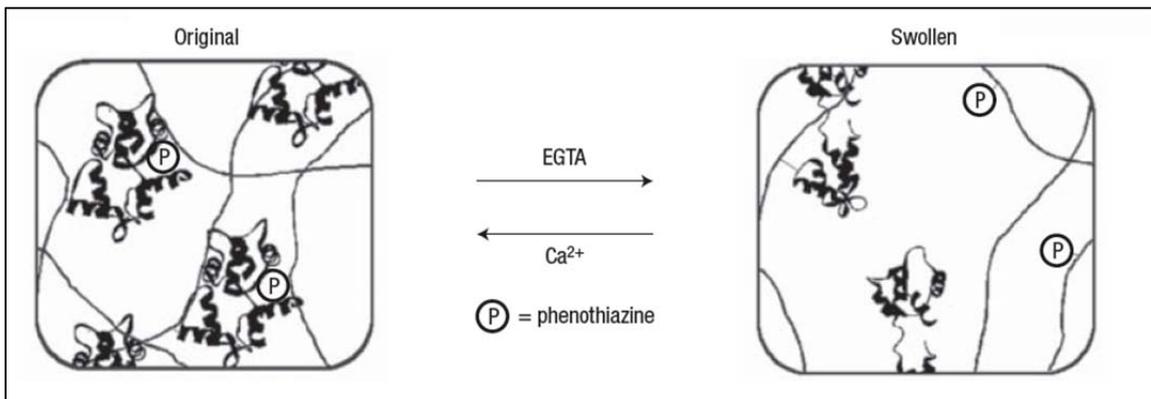
Figure 1.8 A schematic showing swelling and shrinking of calmodulin containing hydrogel in absence and presnece of calcium. Figure reprinted by permission from Nature Publishing Group Ehrick, J. D. *et al. Nat Mater* **4**, 298-302.

oxidized by glucose oxidase. The resulting gluconic acid lowers the pH in the membrane causing the membrane to swell due to the ionization of amine groups, thereby enhancing the permeability of the membrane to insulin (figure 1.9). Hence, permeation of insulin through the membrane is dependent on the concentration of glucose.

In another study an antigen responsive hydrogel was developed by Miyata et al. utilizing chemically engineered antibody and antigen.[100] In this system the antigen, rabbit IgG, and the antibody, goat anti-rabbit IgG, were chemically modified by coupling with N-succinimidylacrylate to introduce vinyl groups in both the antigen and the antibody. The vinyl antibody was copolymerized with acrylamide to create a polymerized antibody that acts as a linear chain in the hydrogel. The hydrogel was then prepared by polymerization of vinyl antigen, acrylamide, N,N'-methylenebisacrylamide as a crosslinker, in the presence of polymerized vinyl antibody. In the presence of free antigen the antigen- antibody hydrogel shows swelling. The swelling mechanism was explained as follows: in the presence of free antigen in the solution containing the antigen-antibody hydrogel, the dissociation of antigen-antibody bonds grafted to the network is induced by the free antigen as a result of the affinity of the antibody for the free antigen being stronger than that for the antigen attached to the hydrogel. The dissociation of antigen-antibody bonds in the hydrogel decreases the crosslinking, thus causing the hydrogel to swell.
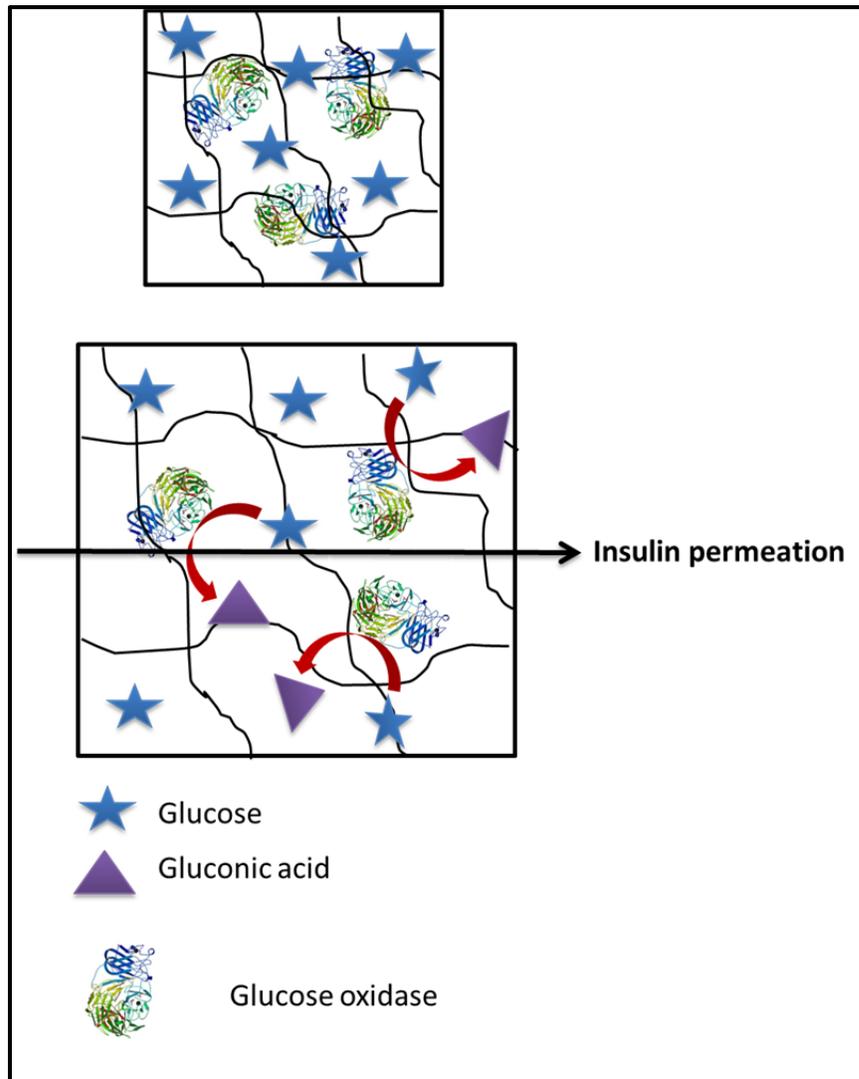
Figure 1.9 pH Sensitive hydrogel with glucose oxidase immobilized within the hydrogel. The hydrogel swells as glucose binds to glucose oxidase and is converted to gluconic acid, allowing insulin permeation. Figure adapted from Ishihara, K, et al, *Polymer Journal* **16**, 625-631 (1984).

Incorporation of Biomaterials into Sensing Devices

As shown in the previous section the integration of proteins with polymers has led to the fabrication of protein-based biomaterials. The incorporation of biomaterials into various platforms has led to the design of devices for a number of applications, including sensing, drug delivery, and clinical diagnostics. Protein biochips are composed of functional protein microarrays immobilized on a solid substrate (glass or silicone). A variety of chips have been designed, including 3D surface, nanowell and plain glass chips. The proteins are either directly immobilized on the solid substrate or captured in polyacrylamide or agarose gel and then immobilized on the solid substrate.[101] Protein biochips find extensive applications in immunoassays,[102] diagnosis,[103] and drug discovery.[104] ProteinChip technology has been utilized to profile and compare protein expression in normal and disease states in the areas of cancer, infectious disease and toxicology.[105]

Nanomaterials are materials that incorporate structures having dimensions in the range 1-100 nm.[106] Many properties of nanomaterials are size-dependent. Nanomaterials are classified on the basis of their morphology as nanotubes, nanowires, nanoparticles (also referred to as quantum dots), and sheet-like two-dimensional structures.[107] A wide variety of biosensing devices based on nanomaterials have been investigated. A highly sensitive electrochemical biosensor for cholesterol has been reported, wherein cholesterol oxidase (ChOx) has been immobilized on well-crystallized flower-shaped ZnO structures composed of hexagonal-shaped ZnO nanorods. These nanorods are then immobilized on gold electrodes. The nanorods based biosensor exhibited a linear dynamic range from

1.0-15.0 μM and low detection limits.[108] In another example, a glucose biosensor was developed by entrapping glucose oxidase (GOD) in a poly(o-aminophenol) (POAP) film for immobilization of the enzyme on a glassy carbon electrode modified with boron-doped carbon nanotubes. The biosensor exhibited high sensitivity, low detection limits (3.6 μM), a short response time of 6 s, satisfactory anti-interference ability, and good stability as the POAP film, which is a non-conducting film, acts as a barrier to prevent electrode fouling from hydrogen peroxide and glucose.[109]

Another interesting example of integration of hybrid materials and devices is provided by microelectromechanical systems (MEMS). These are very small mechanical devices driven by electricity. MEMS devices usually range from 20 μm to mm in size. In a sensor application, an antibody biorecognition element was immobilized on a MEMS cantilever. Binding of the corresponding antigen resulted in a change in the mass which was detected as a change in the frequency of a resonating circuit fabricated as a part of the cantilever structure.[110] In another example, a MEMS thermal biosensor for monitoring metabolites was developed. This sensor consisted of a polymer microfluidic structure integrated with a silicon-based thermal biochip. The enzymes specific for a particular metabolite analyte were immobilized on microbeads in the microfluidic platform.[111]

A further example of incorporation of biospecific elements in sensing devices is represented by fiber optic based sensors, which will be discussed in the following section.

Fiber Optic Biosensors

A fiber optic biosensor is defined as "a biosensor that employs an optical fiber or optical fiber bundle as a platform for the biological recognition element, and as a conduit for excitation light and/or the resultant signal".[112] Fiber optic biosensors can be classified according to either the type of bioreceptor element or the signal transduction method. Some important signal transduction methods include optical, electrochemical, mass sensitive and thermometric methods. The biological components employed in fiber optic biosensors can be enzymes, antibodies or antigens, nucleic acids, whole cells or biomimetic receptors. A fiber optic biosensor can be further classified as:[113]

- Intrinsic sensor – the interaction with the analyte occurs within an element of the optical fiber.

- Extrinsic sensor – the optical fiber is used to couple light to and from the region where the light beam is influenced by the measurement.

Optical fibers transmit light based on the principle of total internal reflection (TIR) (Figure 1.10). An optical fiber is formed by a core with a refractive index n_1 and a cladding with a refractive index n_2. When a ray of light strikes at the boundary of core and cladding a part of light is refracted and a part of it is reflected. If the refractive index of the core is greater than the refractive index of the cladding, i.e., $n_1 > n_2$ and the angle of incidence is larger than the critical angle, then the light is totally reflected internally and propagated through the fiber. The critical angle is defined as the angle of incidence above which TIR occurs. When the incident light is totally reflected internally, a small portion of light penetrates the rarer medium, i.e., the cladding (n_2). This electromagnetic field is

called evanescent wave, and has an intensity that decays exponentially with distance, starting at the interface and extending into the medium of lower refractive index. The evanescent wave can interact with molecules in the penetration depth, thus producing a net flow of energy across the reflecting surface in the medium with lower refractive index to maintain the evanescent field. This transfer of energy leads to attenuation in reflectance called attenuated total reflection (ATR). This phenomenon has been utilized to develop ATP absorption sensors.[114]

Optical fiber biosensors have been used in combination with different types of spectroscopic techniques such as absorption, fluorescence, phosphorescence, and surface plasmon resonance (SPR). Fiber optic bisosensors based on absorbance may consist of the biological component immobilized close to the optical fiber or directly on its surface. The biological component interaction with the analyte results in a change in the absorbance which is related to the concentration of analyte. Light is directed to the optic fiber tip where the biological element is immobilized and the resulting radiation is directed back to the detector by the fiber. Several fiber optic biosensors based on absorbance measurements have been developed for use in medical applications[115, 116] and molecular recognition.[117]

Fluorescence-based fiber optic systems consist of a biological component that is intrinsically fluorescent or is labeled with a fluorescent molecule. In such biosensors the biological components may also be enzymes that catalyze reactions leading to the production or consumption of fluorescent species. For example, dehydrogenases that catalyze nicotinamide adenine dinucleotide (NAD)-dependent reactions have been used for the development of several fiber optic biosensors; these reactions produce NADH or
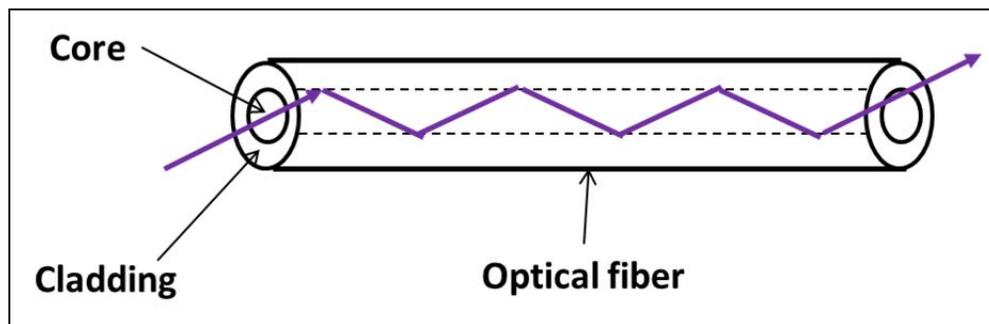
Figure 1.10 Total internal reflection in optical fiber.

NADPH, which can be detected by their fluorescence at $\lambda_{ex} = 350$ nm, $\lambda_{em} = 450$ nm.[118,119] In another example a fiber optic biosensor for glucose based on fluorescence resonance energy transfer was developed.[120] In this system two optical fibers were inserted in a hollow fiber, which was filled with assay solution containing fluorescein isothiocyanate (FITC)-Dextran as the donor and tetramethylrhodamine isothiocyanate (TRITC) labeled ConA (TRITC-ConA) as the acceptor (quencher). In the absence of glucose the donor and the acceptor are significantly close together for the fluorescein signal to be quenched by rhodamine. However, in the presence of glucose the FITC-Dextran is separated from the TRITC-ConA and the fluorescein signal increases in a dose-dependent manner. Hence, this system enables the monitoring of glucose concentrations as a function of changes in fluorescence.

A fluorescent fiber optic immunosensor involves the immobilization of an antibody or antigen on the distal end of the fiber. The incident light introduced at the proximal end of the fiber travels through the fiber via TIR causing excitation of the fluorophore attached to either the antigen or a secondary antibody.[121] A variety of fiber optic based immunosensors for the detection of pesticides have been developed. In an example, a hapten, a derivative of triazine (pesticide), was immobilized on the end of a hard clad silica fiber.[122] The fiber was then incubated with a fluorescently labeled antibody and a sample containing unknown amounts of triazine. This competitive immunoassay is based on measurements of the changes in fluorescence signal to determine the amount of triazine present in a sample.[122]

Immobilization of enzyme based sol-gels on the fiber is another technique for developing fluorescent fiber optic sensors. Doong and coworkers developed a fiber optic

fluorescent biosensor for acetylcholine.[123] In this approach acetylcholinesterase was immobilized in a sol-gel on the distal end of an optical fiber. The acetic acid formed as a result of the enzymatic reaction between acetylcholinesterase and acetylcholine causes a change in the fluorescence intensity of the pH-sensitive fluorescent dye FITC-Dextran. This system was used for the detection of Paraoxon, an organophosphorous pesticide, as Paraoxon inhibits acetylcholinesterase, thus increasing fluorescence.

Surface plasmon resonance (SPR) is a direct optical detection method. SPR is based on TIR, wherein the TIR interface is coated with a thin layer of conducting metal. Polarized light penetrates the metal layer and excites electromagnetic surface plasmon waves propagating within the conductor surface, which is in contact with the low refractive index medium. SPR has successfully been employed in the development of immunosensors for simple and rapid assay of several analytes. Fiber optic SPR sensors are small and suitable for use at remote sites. Several fiber optic SPR sensors have been reported.[124-127] Slavik et al. described the fabrication of a single-mode fiber optic SPR device.[128] The sensing element was created by coating the fiber with a 65-nm layer of gold followed by a 19-nm layer of tantalum pentoxide. The sensor was used for detecting IgG concentrations in nanomolar ranges. Further, in continuation of the above mentioned work, a double layer of anti-Staphylococcal enterotoxin B (SEB) antibody crosslinked by glutaraldehyde was immobilized on the sensor surface for the detection of SEB.[129] A fiber optic SPR immunosensor known as BIAcore probe, which enables dipstick-type sampling, has been launched by the Swedish company BIAcore.[130]

One drawback of fiber optic sensors is the variation in response and signal intensity from fiber to fiber.[113] Immobilization of biological recognition elements on

optical fibers has led to increases in selectivity, sensitivity, and in some cases reversibility.[112] Fiber optic sensors have several advantages, in that they are usually compact and light weight, minimally invasive, and they are not affected by electromagnetic interferences from static electricity, strong magnetic fields, or surface potentials.[112, 113]

STATEMENT OF RESEARCH

The goal of this work was to first create designer proteins with enhanced stability and activity by global incorporation of unnatural amino acids into the structure of GBP and truncated fragments of GBP. Also, modified antibodies were created by photolabeling the unconventional region of the antibody variable region with a fluorophore conjugated nucleotide probe. Secondly, the goal of this work was to develop fiber optic glucose biosensor by using GBP and the unnatural amino acid incorporated proteins covalently attached within a hydrogel and immobilized on a tip of an optical fiber. Also, modified antibodies can be utilized to develop universal biosensing system for various biomolecules since modifying antibodies in the unconventional region does not affect antigen binding. The overall hypotheses for this work have ben formulated below:

- Thermal stability and glucose binding activity of GBP can be altered by global incorporation of unnatural amino acids and these unnatural proteins can be utilized for the development of biosensor for continuous glucose monitoring in physiologically relevant glucose concentrations.

- The newly created glucose-recognition proteins can be incorporated into hydrogel polymer networks, placed at the tip of a fiber optic system and employed in the continuous monitoring of glucose in blood.

- Antibody based biosensing systems/assays can be developed by labeling the unconventional nucleoside binding site with an environment sensitive fluorophore conjugated to a nucleotide/nucleoside probe, and correlating the changes in fluorescence intensity in the presence of the analyte.

GBP has been extensively studied and utilized for developing glucose sensors. However, to the best of our knowledge, no thermally stable GBP or its fragments have been created by global incorporation of unnatural amino acids. Also no glucose biosensor has been developed with unnatural GBP able to detect glucose in physiologically relevant mM ranges. Moreover no fluorescent antibodies have been developed by photolabeling the unconventional region in the variable region of antibodies. It was our belief that global incorporation of unnatural amino acids in GBP would alter the stability and activity of GBP and further modifying the antibodies via fluorophore conjugated nucleotides we can develop universal biosensing system for a variety of biomolecules.

- Chapter Two describes the universal strategy for designing antibody based sensing molecules by labeling an inherent nucleoside binding site. Here as a proof of concept Interleukin 6 was chosen as an analyte to be detected with our modified antibody-based biosensing system.

- Chapter Three describes the development of a biosensing system for monitoring bone loss. Herein, we utilize the biosensing system based on fluorophore modified monoclonal antibody for the detection of Osteonectin, which is a bone biomarker.

- Chapter Four describes the method of global incorporation of unnatural amino acids in GBP and its truncated fragments. It also details the analysis of structure, stability and activity of the unnatural proteins.

- Chapter Five describes the development of fiber optic based biosensor for glucose, wherein, GBP and unnatural amino acid incorporated GBP and GBP fragments are immobilized in a hydrogel.

CHAPTER TWO

UNIVERSAL STRATEGY FOR DESIGNING ANTIBODY BASED SENSING

MOLECULES BY LABELING AN INHERENT NUCLEOSIDE BINDING SITE

The quest for specific and sensitive sensing methods for target molecules is an on-going challenge. While the utility of biosensors has been proven, their development has been limited to the availability of natural or artificial receptors that recognize the target molecule. Herein, a universal approach is demonstrated for the design of antibody based sensing molecules that overcome this limitation. In particular, the antibody is modified by covalently attaching a nucleoside probe containing an environment-sensitive fluorophore to the inherent non-canonical nucleoside-binding site of the antibody. The non-canonical nucleoside binding site resides between the light and the heavy chains of the variable domain of antibodies, and tethering nucleoside probes to this site does not affect antigen binding.[131, 132] The binding of the antigen to the antibody causes a change in the microenvironment of the nucleoside-binding site that is manifested by an alteration in the fluorescence properties of the fluorophore in a manner that is related to the concentration of antigen present in the sample. This relationship constitutes the basis for the development of the antibody based assays and biosensing systems.

Antibodies have found wide applicability in a range of immunoassay[127, 133, 134] and biosensor[135-137] formats. Fluorescent antibodies with a potential to be used as biosensors have been developed, wherein, a fluorescent stilbene derivative is bound to the antigen binding site[138-141]. Besides the antigen binding site, an inherent non-canonical site is

present in the variable domain of immunoglobulin (Ig) formed by invariant residues of both light and heavy chains of Ig, which binds purine containing nucleoside photoaffinity probes with high affinity.[1] The purine ring is held via base-stacking interactions within the non-canonical site, while the ribose ring is exposed to the surface of the molecule allowing a number of reporter moieties to be tethered to the antibody.[131, 132] Docking to this unconventional site does not interfere with antigen binding.[2] Amino acids in the non-canonical site that make contact with the nucleotide moiety include Tyr from the light chain, as well as Asp and Trp from the heavy chain.[1] To demonstrate the prevalence of these amino acids within the non-canonical site, the sequences of a series of IgGs were aligned. The sequences of 740 Fab antibody fragments were obtained from the Protein Data Bank by searching with the keyword "Fab antibody" (search conducted on 20th October 2010).The sequences were then sorted by their release date. The most recently released 100 Fab sequences thus obtained with both the heavy and the light chains were aligned using T-Coffee software.[142] The sequence alignment confirms the presence of the three invariant amino acids in the light and heavy chains of antibodies. Herein, we describe a reagentless (i.e., no need for use/addition of reagents) biosensing system based on an antibody labeled with a fluorescent nucleotide probe (Figure 2.1). This strategy is universal as it can be used for the detection of any given molecule provided the availability of a specific antibody toward this target molecule.

To demonstrate the feasibility of this approach, we utilized an anti-interleukin 6 (IL-6) monoclonal antibody that binds selectively to IL-6. IL-6 is a multifunctional cytokine consisting of 185 amino acids secreted by T cells and macrophages.[143] IL-6 promotes inflammatory events through activation of T cells, differentiation of B cells,
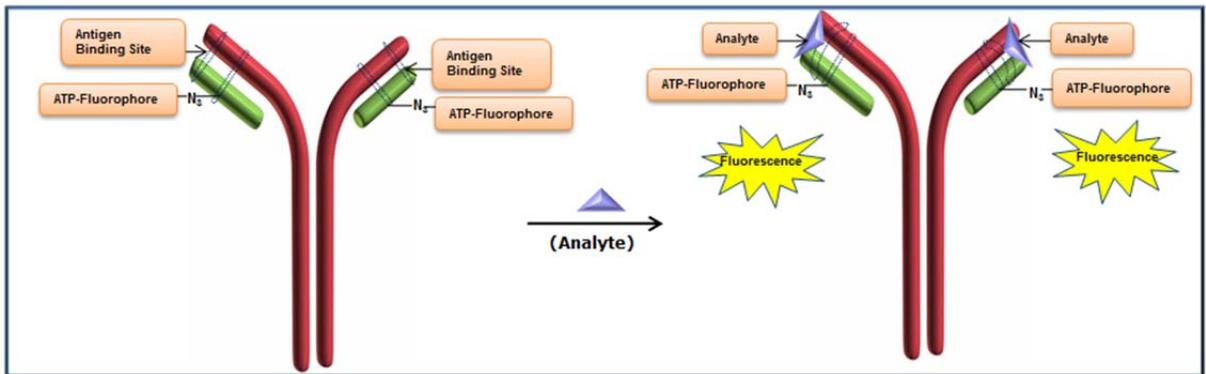
FIGURE 2.1 Schematic showing the strategy for development of biosensing system based on fluorophore labeled monoclonal antibody

and the induction of acute phase reactants by hepatocytes.[144, 145] In addition, IL-6 plays a protective role during disease, acting both as a pro- and an anti-inflammatory cytokine.[146] Elevated concentrations of IL-6 in body fluids have been reported in various disease states such as cardiac myxomas and cardiovascular diseases[147-149] and is employed as a general marker of inflammation.[150]

EXPERIMENTAL PROCEDURES

Materials

Mouse anti-IL-6 monoclonal antibody and recombinant Human IL-6 were purchased from USBiological (Massachusetts, MA). Nucleotide probes 8-N$_3$ATP and 2-N$_3$ATP were obtained from Affinity Labeling Technologies (ALT) Corporation (Lexington, KY). Alexa Fluor 594 cadaverine was obtained from Invitrogen (Eugene, OR). The Bradford protein assay kit was obtained from Bio-Rad laboratories (Hercules, CA). Carry Eclipse Spectrophotometer (Milpitas, CA) was used for fluorescence measurements.

Methods

Selecting the photoaffinity probe

Two radioactive nucleotide probes, [γ-^{32}P]8-N$_3$ATP and [γ-^{32}P]2-N$_3$ATP were evaluated for their reactivity with mouse anti-interleukin 6 monoclonal antibody. For the labeling procedure 8 μg of antibody was incubated with 300 μM of each probe in two

different tubes in a final volume of 62 μL on ice for 10 min. After 10 min incubation, the

two tubes were photolyzed with a hand-held UV lamp at 254 nm for 2 min, while the

reaction mixture was agitated in-between to prevent local heating. The reaction was

quenched by adding protein solubilizing mixture (10% SDS, 3.6 M urea, 162 mM DTT,

0.0025% Pyronin Y, 20 mM Tris, pH 8.0) and heating it in a water bath for 5 min. The

reaction products were then analyzed by SDS PAGE. ^{32}P incorporation was detected by

autoradiography.

Saturation of labeling

An amount of 2 μg of mouse anti-interleukin 6 monoclonal antibody was

incubated with increasing concentrations of [γ-^{32}P]2-N$_3$ATP for 20 min on ice and

photolyzed with a hand-held lamp at 254 nm for 75 s. The reaction was quenched by

adding 5 μL of 0.25 mg/mL cysteine and analyzed by SDS/PAGE and autoradiography.

Conjugation of 2-N$_3$ATP to Alexa Fluor 594 cadaverine

The conjugation of 2-N$_3$ATP to the fluorophore was done at Affinity Labeling

Technologies. A 1:1 ratio of 2-N$_3$ATP and Alexa Fluor 594 cadaverine (Invitrogen) was

added to 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 5 mM sulfo-N-

hydroxysulfosuccinimide and allowed to react at room temperature for 2 h. The conjugate

was then purified using a diethylaminoethyl cellulose column and an elution gradient of 0

-300 mM triethylammonium carbonate.

Determination of K_D of the monoclonal antibody for 2-N$_3$ATP-fluorophore

      Competitive labeling of the anti-interleukin 6 (IL-6) monoclonal antibody with 2-N$_3$ATP [γ ^{32}P] and 2-N$_3$ATP-fluorophore was done. 2-N$_3$ATP [γ ^{32}P] (30 µM) and 2 µg of the antibody was added to each 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM and 120 µM of 2-N$_3$ATP-fluorophore tube and incubated for on ice 20 min. The tubes were then photolyzed for 75 s. The reaction was quenched by adding 5 µL of 0.25 mg/mL cysteine and analyzed by SDS/PAGE and autoradiography. The apparent K_D was determined using GraphPad Prism 5 software.

Conjugation of the antibody to the 2-N$_3$ATP-fluorophore

      For the conjugation of 2-N$_3$ATP-Alexa Fluor 594 cadaverine to the antibody, 4 µg of the antibody was incubated with 30 µM 2-N$_3$ATP labeled fluorophore in a final volume of 50 µL for 20 min, on ice and in the dark. The reaction was photolyzed with a hand-held UV lamp at 254 nm for 4 min. To quench the photolytic reaction, cysteine (10 µL of 1mg/ml) was added. The unbound fluorophore was separated from the antibody bound fluorophore by dialyzing against six changes of 10 mM phosphate buffer, pH 7.0 over 24h.

Fluorescence studies of the interaction of the labeled antibody with IL-6

      The effect of IL-6 binding on the fluorescence signal of the label attached to the non-canonical site of the IL-6 antibody was studied by incubating 4 µg/mL of the labeled antibody with different concentrations of IL-6 for 30 min at room temperature in the dark. The sample solutions were prepared using 10 mM phosphate buffer, pH 7.0. The

fluorescence intensity of the samples (total volume 200 µL) was measured on a Cary Eclipse Spectrofluorometer.

RESULTS AND DISCUSSION

Nucleotide probes conjugated with a fluorophore can be docked to the non-canonical nucleoside binding site of the antibody without interfering with binding of antigen to the canonical antigen-binding site. The mouse anti-human IL-6 monoclonal antibody was labeled with two photoreactive probes, [γ-^{32}P]-2-N$_3$ATP (Figure 2.2a) and [γ-^{32}P]-8-N$_3$ATP (Figure 2.2b) to test their reactivity toward the antibody. Both the light and heavy chains were labeled with the radioactive probe indicating that the nucleoside binding site is composed of amino acids located on both chains (Figure 2.3a). It was also determined that [γ-^{32}P]-2-N$_3$ATP labels the antibody better than [γ-^{32}P]-8-N$_3$ATP (Figure 2.3b). Further, to optimize the efficiency of labeling the antibody with the photoreactive probe, the concentration of the probe required to label the antibody was determined by incubating anti-IL-6 monoclonal antibody with increasing concentrations of [γ-^{32}P]-2-N$_3$ATP followed by exposure to UV light to activate the photolabel (Figure 2.4). The concentration of probe selected to label the antibody in all subsequent experiments was ~25 µM, as this is relatively low concentration and generates a good signal.

After proving that [γ-^{32}P]-2-N$_3$ATP labeled the antibody successfully, a fluorescent, rather than radiolabeled derivative of 2-N$_3$ATP was synthesized. In that regard, Alexa Fluor 594 cadaverine was selected for coupling to 2-N$_3$ATP. This
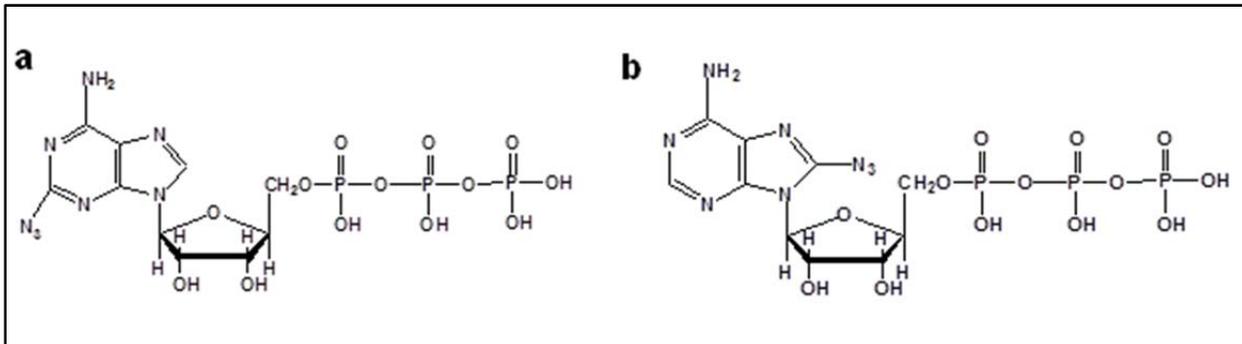
Figure 2.2 Structures of nucleotide probes utilized for photolabeling of antibody. (a) [γ^{32}P] 2-N$_3$ATP (b) [γ-^{32}P] 8-N$_3$ATP
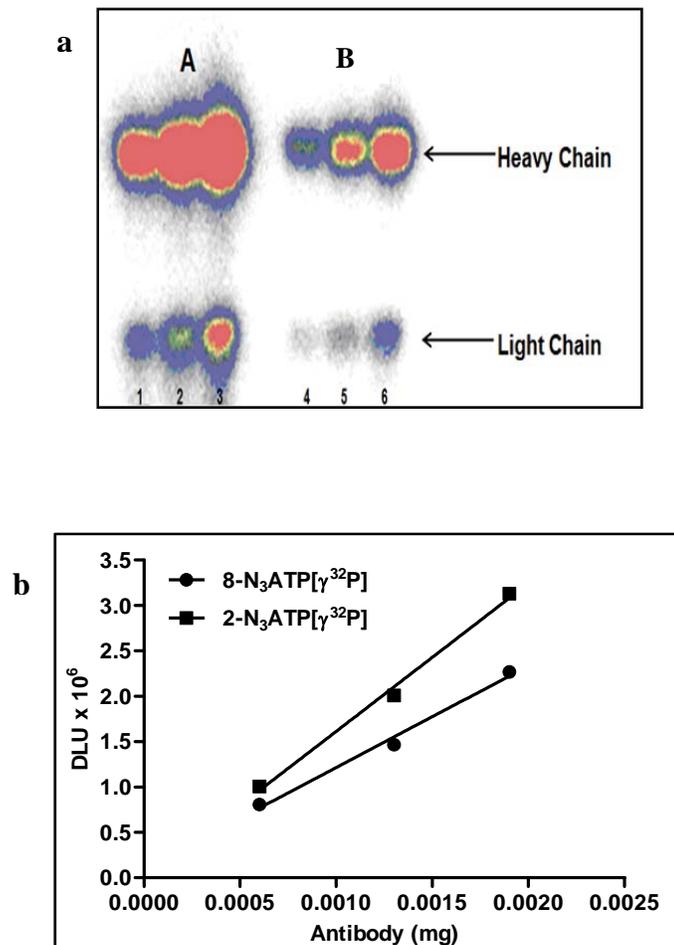
Figure 2.3 Antibody labeled with radioactive nucleotide probes. (a) autoradiogram of the antibody labeled with the photoreactive probes [γ-^{32}P] 2-N$_3$ATP (A) and [γ-^{32}P] 8-N$_3$ATP (B). Lanes 1, 4; 2, 5; and 3, 6 correspond to the use of 0.6 μg, 1.3 μg and 1.9 μg of labeled antibody, respectively. (b) Normalized data obtained from the autoradiogram of antibody labeled with the photoreactive probes.
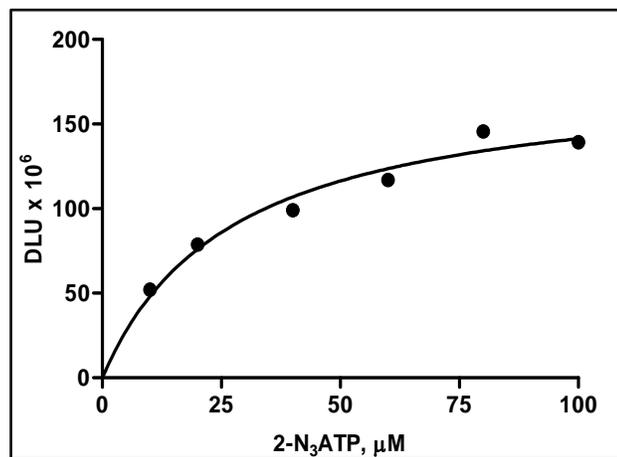
Figure 2.4 Saturation of photolabeling of antibody with [γ-^{32}P] 2-N$_3$ATP.

fluorophore has long excitation and emission wavelengths (590 nm/617 nm), thus circumventing interferences from common fluorescent compounds present in biological samples. The amine functional group of Alexa Fluor 594 cadaverine was utilized for conjugation to 2-N$_3$ATP, since it was observed that 2-N$_3$ATP labels the antibody with a greater efficiency than the 8-N$_3$ATP *vide supra*. The apparent K_D for 2-N$_3$ATP-Alexa Fluor 594 (Figure 2.5) cadaverine to the non-canonical site was determined to be ~17 μM. The fluorescently labeled nucleotide was further conjugated to the mouse anti-human IL-6 monoclonal antibody at the non-canonical site upon exposure to UV light.

The effect of antigen (IL-6) binding on the fluorescence signal of the 2-N$_3$ATP-Alexa Fluor 594 (Figure 2.6a) labeled antibody was evaluated. The apparent K_D for IL-6 to the fluorophore labeled antibody was determined to be ~2.4 x 10^{-8} M. It was observed that binding of IL-6 to the antibody resulted in an increase of the fluorescence intensity of the fluorophore with increasing concentration of antigen (Figure 2.6b). The experiments suggest that binding of IL-6 to the antigen binding site induces a change in the conformation of the non-canonical site causing a change in the microenvironment surrounding the fluorophore, which is manifested by a change in the fluorescence signal. Because the site in the antibody where the fluorescent probe is bound is adjacent to the antigen-binding site in the variable region of the antibody, there is an allosteric effect that upon antigen binding alters the fluorescence signal of the fluorophore.
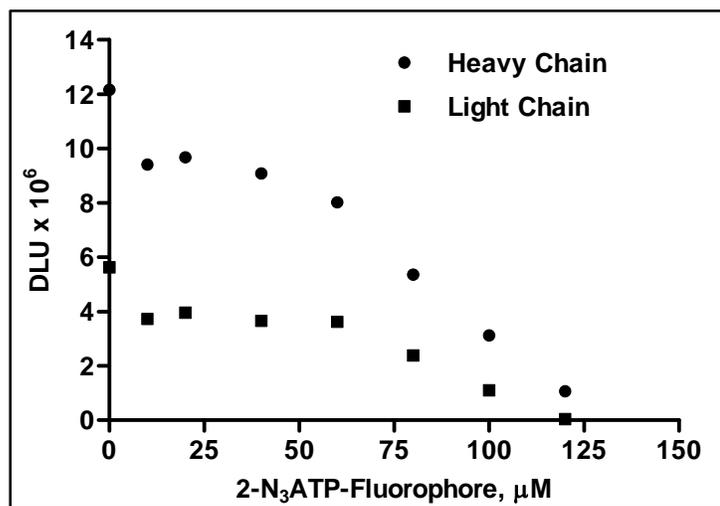
Figure 2.5 Competitive labeling of anti-interleukin 6 (IL-6) monoclonal antibody with 2-N$_3$ATP -Alexa Fluor 594 cadaverine and 2-N$_3$ATP [γ ^{32}P].
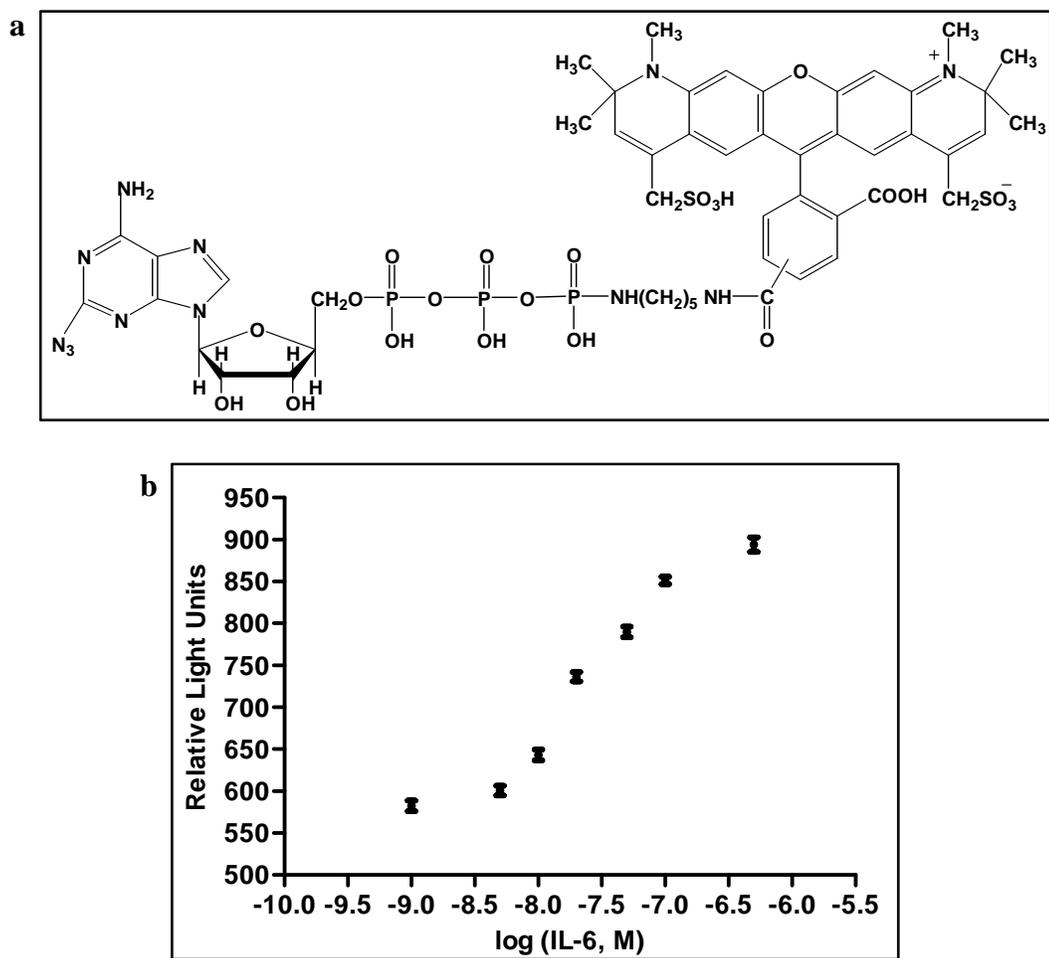
Figure 2.6 Structure of fluorescent nucleotide probe and the effect of Il-6 on the fluorescence intensity of the probe. (a) 2-N$_3$ATP-Alexa Fluor 594 cadaverine (b) Dose-response curve for IL-6. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.

CONCLUSIONS

Herein we have developed a universal method for the design of biosensors. We demonstrated that a fluorophore-modified nucleotide probe can bind to the non-canonical site of the antibody without affecting antigen binding. Additionally, we have shown that binding of the antigen to the antibody alters the fluorescence intensity of the fluorophore-modified nucleotide probe in an allosteric fashion. The change in fluorescence intensity of the label can be correlated to the concentration of IL-6 in the sample. Because of the widespread nature of this non-canonical site among IgGs, and availability of antibodies for practically any molecule of interest, this versatile biosensing approach should find wide applicability in a variety of fields where detection of antigen-antibody interactions is important. Among those, we can envision their use in sensing in a variety of samples, imaging, localization of target molecules in cells, etc.

CHAPTER THREE

BIOSENSING SYSTEM BASED ON MODIFIED ANTIBODY FOR

MONITORING BONE LOSS

INTRODUCTION

Bone undergoes mass changes throughout life, also referred to as bone remodeling. Bone remodeling involves cycles of bone deposition by osteoblasts and bone resorption by osteoclasts.[151] Bone remodeling allows for the renewal of bones in vertebrates. Bone is continuously remodeled in response to mechanical and physiological stress.[152] Bone mass is maintained by a balance between the rates of bone deposition and bone resorption.[151] However, when the rate of bone resorption is faster than the rate of bone deposition it leads to a disorder called osteoporosis.[151] Osteopenia is another bone disorder where bone mass is less than average. It is sometimes also referred to as onset of osteoporosis. Besides these health conditions, bone mass loss is also reported in humans after prolonged space flights and weightlessness or microgravity.[153, 154] Although the mechanism of microgravity induced bone loss is not fully known, it has been attributed to a decrease in osteoblast function without a significant change in bone resorption.[154] Several ground based studies on microgravity induced bone loss have been performed using various models. These studies suggest that bone loss may occur because of inhibition of osteoblast differentiation from human mesenchymal stem cells due to a failure in cellular expression of osteonectin.

Osteonectin is a 32,000 kDa glycoprotein occurring in the noncollagenous matrix of bone, extracellular matrices and some body compartments including blood platelets.[155,]

[156] Hence, two forms of ON are known, a bone derived form and a platelet derived form. Osteonectin is a major constituent of basement membrane matrix of many embryonic and adult tissues.[157] Osteonectin contains structural domains including an acidic domain, which is rich in aspartic and glutamic acid, a NH_2-terminal domain, a cysteine rich domain with potential glycosylation sites, an alpha helical domain, and two calcium binding motifs.[157, 158] ON binds to type I collagen and hydroxyapatite and, consequently, plays a key role in initiating mineral deposition and linking mineral to the matrix.[159] Osteonectin deficient mice have shown decreased bone mineral density and a decreased function of osteoblasts and osteoclasts resulting in osteopenia.[151] Several animal models of bone fragility show a decreased expression of osteonectin. Further, osteonectin has also been determined as a salivary biomarker of alveolar bone loss.[160, 161] Herein, we present an antibody based biosensing system for osteonectin that could find application in the prevention and treatment of bone loss for healthier life. Our biosensing system is based on the previously developed concept of conjugating a fluorophore labeled nucleotide at the non-canonical site of an antibody in which change in the conformation induced by the binding of antigen at the antigen binding site causes a change in fluorescence intensity. Specifically, we used a monoclonal antibody against osteonectin, which was labeled with 2-N_3ATP conjugated to Alexa Fluor 594 cadaverine. The intensity of fluorescence of the label was found to increase with increasing osteonectin concentration in the sample.

EXPERIMENTAL PROCEDURES

Materials

Anti-human osteonectin/SPARC monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant osteonectin was purchased from ALPCO Diagnostic (Salem, NH). Nucleotide probes 8-N_3ATP and 2-N_3ATP were obtained from Affinity Labeling Technologies (ALT) Corporation (Lexington, KY). Alexa Fluor 594 cadaverine was obtained from Invitrogen (Eugene, OR). The Bradford protein assay kit was obtained from Bio-Rad laboratories (Hercules, CA). Carry Eclipse Spectrophotometer (Milpitas, CA) was used for fluorescence measurements

Methods

Selecting the photoaffinity probe

Two radioactive nucleotide probes, [γ-^{32}P]8-N_3ATP and [γ-^{32}P]2-N_3ATP were evaluated for their reactivity with mouse anti-osteonectin monoclonal antibody. For the labeling procedure 8 μg of antibody was incubated with 358 μM of each probe in two different tubes in a final volume of 58 μL on ice for 10 min. After 10 min incubation, the two tubes were exposed to UV light with a hand-held UV lamp at 254 nm for 75 s, while the reaction mixture was agitated in-between to prevent local heating. The reaction was quenched by adding a protein solubilizing mixture (10% SDS, 3.6 M urea, 162 mM DTT, 0.0025% Pyronin Y, 20 mM Tris, pH 8.0) and heating it in a water bath for 5 min. The reaction products were then analyzed by SDS PAGE. ^{32}P incorporation was detected by autoradiography.

Conjugation of 2-N$_3$ATP to Alexa Fluor 594 cadaverine

The conjugation of 2-N$_3$ATP to the fluorophore was performed at Affinity Labeling Technologies. A 1:1 ratio of 2-N$_3$ATP and Alexa Fluor 594 cadaverine was added to 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 5 mM sulfo-N-hydroxysulfosuccinimide and allowed to react at room temperature for 2 h. The conjugate was then purified using a diethylaminoethyl cellulose column and an elution gradient of 0 -300 mM triethylammonium carbonate.

Conjugation of the antibody to the 2-N$_3$ATP-fluorophore

For the conjugation of 2-N$_3$ATP-Alexa Fluor 594 cadaverine to the antibody, 0.2 mg/mL of the antibody was incubated with 90 μM 2-N$_3$ATP labeled fluorophore in a final volume of 100 μl for 20 min, over ice and in dark, in two flat bottom eppendorf tubes. The reaction mixture was exposed to UV light with a hand-held UV lamp at 254 nm for 2 min. To quench the photolytic reaction, cysteine (12.5 μL of 1mg/mL) was added. The unbound fluorophore was separated from the antibody bound fluorophore by dialyzing against six changes of 10 mM phosphate buffer, pH 7.0 over 24h. The concentration of fluorophore-labeled antibody (ON antibody-2-N$_3$ATP-Alexa Fluor 594 cadaverine) was determined by Bradford assay.

Calibration curve for osteonectin antibody-2-N$_3$ATP-Alexa Fluor 594 cadaverine

In order to generate calibration curves for the ON antibody-2-N$_3$ATP-Alexa Fluor 594 cadaverine serial dilutions were made of fluorophore-labeled antibody from a stock solution at concentration of 6.7 x 10^{-7} M with 10 mM phosphate buffer, pH 7.0.

Fluorescence measurements were performed in a microtiter plate with 100 μL samples. The samples were excited at 590 nm and the emission was measured at 617 nm. A volume of 100 μL of 10 mM phosphate buffer, pH 7.0, was used as blank. The fluorescence intensity of the samples was measured on a Cary Eclipse Spectrofluorometer.

Association study

A volume of 50 μL of 3 x 10^{-8} M fluorophore-labeled antibody and 50 μL of varying concentrations of osteonectin ranging from 1 x 10^{-7} M to 1 x 10^{-10} M were incubated in a microtiter plate for a time period ranging from 5 to 60 min at room temperature. The fluorescence of the samples in microtiter plate were measured upon excitation at 590 nm after every 5 min for 30 min and every 15 min from 30 to 60 min. A sample of 100 μL fluorophore-labeled antibody was used as a control and 10 mM phosphate buffer, at pH 7.0, was used as blank.

Fluorescence studies of the interaction of the labeled antibody with osteonectin

The effect of ON binding on the fluorescence signal of the label attached to the non-canonical site of the ON antibody was studied by incubating 4 μg/mL of the labeled antibody with different concentrations of ON for 30 min at room temperature in the dark. The sample solutions were prepared by mixing 100 μL of antibody solution and 100 μL of ON solution. The solutions were made in 10 mM phosphate buffer, pH 7.0. The fluorescence intensity of the samples (total volume 200 μL) was measured on a Cary Eclipse Spectrofluorometer.

Selectivity study

In order to determine whether the monoclonal antibody based sensor is selective for osteonectin, 4 μg/mL fluorophore labeled antibody was incubated with different concentrations of osteonectin and inteleukin-6 (IL6) separately in a total volume of 200 μL in a microtiter plate for 25 min in dark at room temperature. The fluorescence of the samples was measured upon excitation at 590 nm. A sample of 100 μL fluorophore-labeled antibody was used as a control and 10 mM phosphate buffer, pH 7.0, was used as blank.

RESULTS AND DISCUSSION

An anti-osteonectin monoclonal antibody was labeled with two radioactive nucleotide probes, $[\gamma-^{32}P]$-2-N_3ATP and $[\gamma-^{32}P]$-8-N_3ATP to determine the reactivity of the probes against the osteonectin antibody. The photolabeld osteonectin antibody was analyzed via SDS PAGE analysis to determine which nucleotide probe labels the antibody better. The determination of ^{32}P incorporation via autoradiography indicates that both the light and heavy chains of osteonectin antibody are labeled with the radioactive probe. Hence, the nucleotide binding site resides within the light and heavy chain of antibody. Autoradiogram data (Figure 3.1) also show that $[\gamma-^{32}P]$-2-N_3ATP labels the antibody better than $[\gamma-^{32}P]$-8-N_3ATP. Therefore, for further experiments the osteonectin antibody was labeled with the fluorescent derivative of 2-N_3ATP.
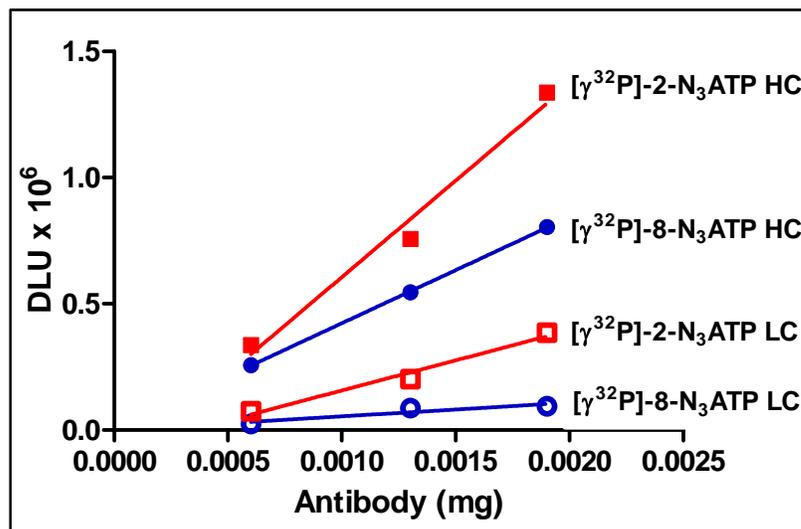
Figure 3.1 Data obtained from autoradiograph of monoclonal antibody against osteonectin with $[\gamma\text{-}^{32}P]8\text{-}N_3ATP$ and $[\gamma\text{-}^{32}P]2\text{-}N_3ATP$. HC= Heavy Chain, LC=Light Chain.

In our biosensing system, we utilized the non-canonical nucleotide binding site to dock a fluorescent reporter molecule covalently attached to 2-N$_3$ATP. We utilized Alexa Fluor 594 cadaverine as the fluorescent reporter molecule, because it has long excitation and emission wavelengths (590 nm/617 nm), thus circumventing interferences from common fluorescent compounds present in biological samples. The amine functional group of Alexa Fluor 594 cadaverine was utilized for conjugation to 2-N$_3$ATP. Alexa Fluor 594 cadaverine labeled 2-N$_3$ATP was further conjugated to the osteonectin monoclonal antibody at the non-canonical site upon exposure to UV light. Haley and coworkers have previously determined via computer modeling that the nucleoside base is sandwiched between the rings of an invariant Trp in the heavy chain and Pro in the light chain forming the non-canonical binding site.[131] The azido group of the purine ring on the other hand forms a covalent bond with the amino acids in the light chain of antibody upon irradiation with UV light[131]. The unbound nucleotide fluorophore conjugate to the ON antibody was separated from the fluorophore-bound antibody by dialysis, and the protein concentration of the fluorophore-labeled antibody was determined by Bradford assay.

The amount of fluorophore-labeled antibody required for further experiments was determined by generating calibration curves that relate the amount of fluorescent signal to the different concentrations of the fluorescent antibody (Figure 3.2). The concentration of fluorescent antibody selected for further experiments was 1 X 10^{-8} M as this was a relatively low concentration that generates a good detectable signal.

To determine the amount of time required to obtain the maximum possible change in the signal upon incubating the fluorescent osteonectin antibody with osteonectin, an
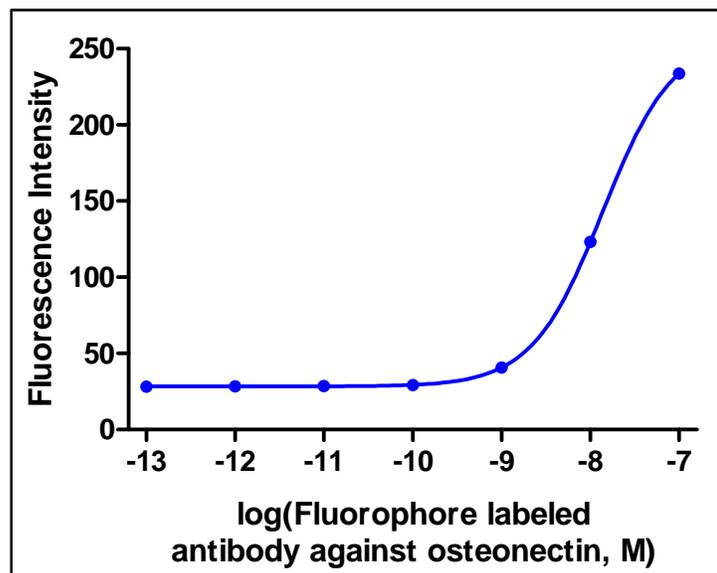
Figure 3.2 Calibration curve for 2-N$_3$ATP-Alexa Fluor 594 labeled antibody against osteonectin. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.
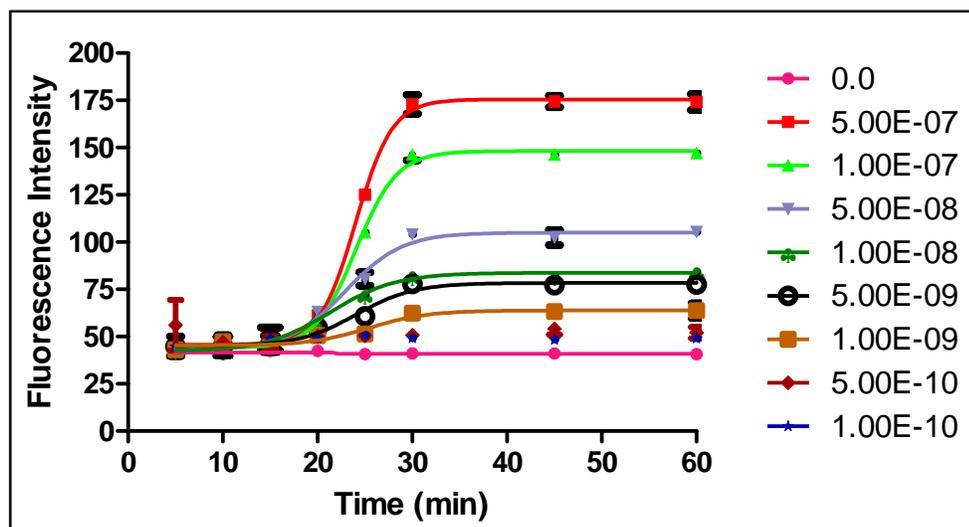
Figure 3.3 Association curve for 2-N$_3$ATP-Alexa Fluor 594 labeled antibody against osteonectin with osteonectin. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.

association study was performed. Figure 3.3 shows the association curve for fluorescent osteonectin antibody with osteonectin. A maximum increase of fluorescence intensity was obtained after 30 min incubation of fluorescent antibody and antigen. However, a distinguishable increase of fluorescence intensity was observed after 25 min incubation of antibody and antigen. The time study shows a lag phase over the first 15 min of incubation of the analyte (antigen) with the fluorophore labeled antibody. This lag phase can be because of the time required for the diffusion of the analyte in the binding site of the antibody or the conformation change in the binding site in the presence of the analyte. Therefore, for all subsequent experiments an incubation time of 25 min was used.

A dose-response curve (Figure 3.4) was generated to evaluate the effect of antigen (osteonectin) binding on the fluorescence signal of the 2-N_3ATP-Alexa Fluor 594 labeled antibody. An increase in the fluorescence signal was observed with increasing concentration of antigen. The change in the fluorescence is attributed to the change in the microenvironment surrounding the fluorophore induced by the change in the conformation of the non-canonical site to which the fluorophore is bound. As previously described by Haley and coworkers this non-canonical site resides adjacent to the antigen binding site in the variable region of the antibody. Therefore, there is an allosteric effect that upon antigen binding alters the fluorescence signal of the fluorophore.

To evaluate the selectivity of our biosensing system for osteonectin, selectivity studies were done by incubating the fluorophore-labeled osteonectin antibody with varying concentrations of osteonectin and IL6 for 25 min and the change in fluorescence intensity was measured. Figure 3.5 shows an increase in the fluorescence intensity of osteonectin antibody with increasing concentrations of osteonectin. However, no change
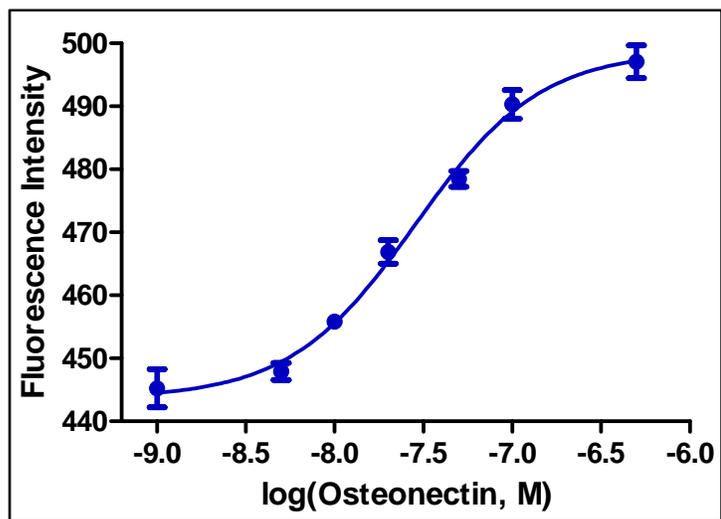
Figure 3.4 Dose-response curve for osteonectin. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.
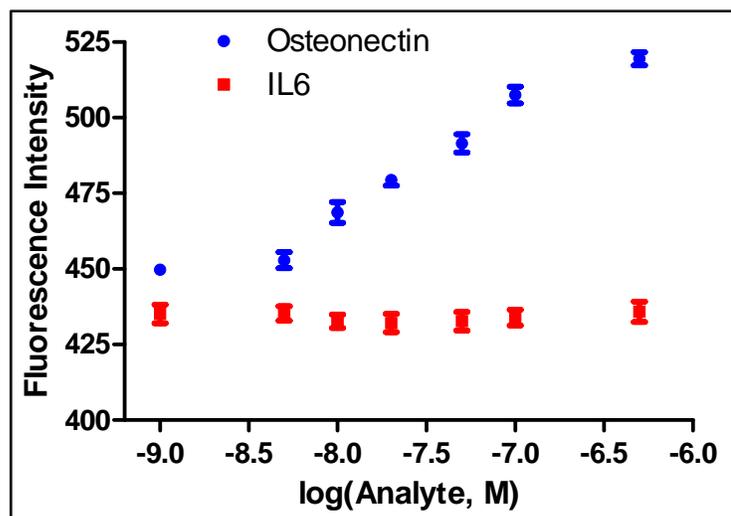
Figure 3.5 Selectivity studies for 2-N$_3$ATP-Alexa Fluor 594 labeled antibody against osteonectin with osteonectin and IL6. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.

in the fluorescence intensity is observed for osteonectin antibody with increasing concentrations of IL6. This confirms that fluorophore-labeled osteonectin antibody binds specifically to osteonectin and hence, the biosensing system is very specific for the antigen.

CONCLUSIONS

In conclusion we have demonstrated the use of our previously developed universal biosensing system for the detection of osteonectin based on labeling the non-canonical nucleoside binding site of an anti-osteonectin antibody. We have also demonstrated that our biosensing system based on fluorescently labeled antibody is very selective for the antigen. Our biosensing system will help in the clinical analysis of osteonectin. Also this type of biosensing system can be extended to different kinds of analytes of interest.

CHAPTER FOUR

Designer Glucose Recognition Proteins via Global Incorporation of Unnatural

Amino Acids

INTRODUCTION

A key advancement in biotechnology has been the ability to prepare designer proteins with a wide spectrum of chemical and physical properties. The properties of these proteins have been tailored, thus altering their function and stability, by employing a variety of chemical and genetic methods including the incorporation of unnatural amino acids into the protein structure[162-166]. Rather recently, it has been reported that the incorporation of unnatural amino acids allows to tune the properties and thermal stability of proteins.[72, 167] Several fluorinated non-natural amino acids such as fluorovaline,[168] fluoroleucine,[167-169] fluoroisoleucine,[170] fluorophenylalanine[171] and fluoroproline[172] have shown to enhance thermal stability and resistance to chemical denaturation. Since fluorine has similar Van der Walls radii as hydrogen, it can be replaced in amino acids with minimal steric perturbation of the protein.[173] Fluorine in high electronegative and hydrophobic amino acids confers distinct properties in proteins. Thus, adopting such a strategy could endow proteins with improved properties, increased temperature stability, increased shelf-lives, a decrease in refrigeration storing needs, and easier packaging/transport of these reagents. In addition, it would allow for their use at RT and, potentially, in extreme environments.

GBP has been proposed as a recognition element in biosensors for the detection of glucose in a wide variety of fields, including in the management of diabetics.[174, 175] GBP is a 33 kDa periplasmic binding protein[39] with two similarly folded globular domains connected via three protein segments.[10] The sugar-binding site is located within a cleft between the two domains of the protein. Upon glucose binding, a conformational change in the protein at the hinge region occurs. In the open form of GBP (in the absence of glucose) the two domains are far apart and the cleft is exposed to the solvent, while in the closed form the glucose is engulfed within the cleft.[10] Construction of GBP-based fluorescent biosensors by covalently coupling organic chemical fluorophores to various amino acids introduced in the protein via mutagenesis, in or around the glucose binding pocket has been reported by us and others.[10, 176, 177] Although these chemically-modified GBPs demonstrated sensitive and selective response to glucose, the need for highly stable sensors for long-term detection of glucose in hypoglycemic, normal and hyperglycemic ranges continues to be a challenge.

Herein, we demonstrate the production of unnatural glucose recognition proteins (GRPs) by global incorporation of the unnatural amino acids 5-fluorotryptophan (FW) (Figure 4.1 a) and 5,5,5-trifluoroleucine (FL) (Figure 4.1b) within the structure of a genetically engineered GBP, GBP152, of *E.coli* and its truncated fragments (GRP1 and GRP2).[42] In our work we replaced the natural leucines and tryptophans in GBP152 and its truncated fragments with FL and FW, and studied their structure, activity, and stability. Far-UV circular dichroism (CD) of GRPs-FW/FL revealed change in the secondary structure compared to GBP152, indicating that the incorporation of unnatural amino acids alter the secondary structure of proteins. Unnatural GRPs could still bind to
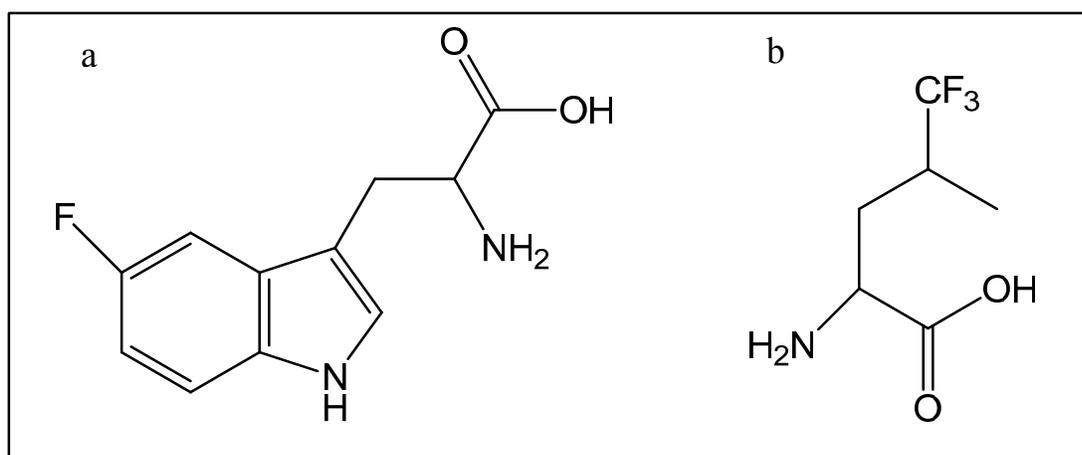
Figure 4.1 Chemical structures of unnatural amino acids incorporated in glucose recognition protein (a) 5-fluorotryptophan (b) 5,5,5-trifluorotryptophan

glucose; however their binding constants are affected by incorporation of unnatural amino acids. In contrast, temperature-induced CD changes showed an increase in the thermal stability of almost all GRPs-FL/FW except GRP1,2-FW, whose thermal stability remains unaltered.

EXPERIMENTAL PROCEDURES

Materials

M9 minimal salts was purchased from Difco^{TM} (Sparks,MD) Ampicillin sodium salt, anhydrous calcium chloride, magnesium sulfate, calcium chloride, thiamine, 5-fluorotryptophan, and 5,5,5-trifluoroleucine were purchased from Sigma-Aldrich (St. Louis, MO). Imidazole was purchased from J.T. Baker, Inv. (Phillipsburg, NJ). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Gold Biotechnology, Inc. (Houstan, TX). The Bradford protein assay kit was purchased from Bio-Rad laboratories, Inc. (Hercules, CA). Sodium Dodecyl Sulfate (SDS) was purchased from Curtin Mathesin Scientific, Inc. (Houstan, TX). Nickel-NitriloTriAcetic acid (Ni-NTA) agarose was purchased from Qiagen (Valencia, CA). Tris glycine SDS Page gels and 7-diethylamino-3-(((((2-maleimidyl)ethyl-amino)carbonyl)coumarin (MDCC) and YM-10 Microcon centrifugal devices were purchased from Invitrogen (Carlsbad, CA). D-Glucose, anhydrous was purchased from Spectrum (New Brunswick, NJ).) HEPES was purchased from VWR International (Bridgeport, NJ). All solutions were prepared using deionized reverse osmosis (MILL-Q Water purification System, Millipore, Bedford, MA) water.

Apparatus

Cell cultures were grown on an orbital shaker from Forma Scientific (Marrietta, OH) and a Beckman J2-MI centrifuge (Palo Alto, CA) was used for harvesting the cells. GRPs-FW/FL were obtained by lysing the cells with a 550 Sonic Dismembrator from Fisher Scientific. Fluorescence studies of GRPs-FW/FL in solution were performed on Varian Cary Eclipse fluorescence Spectrophotometer. CD measurements were performed with a Jasco model J-810 spectropolarimeter (JASCO Ltd. UK).

Methods

Expression and purification of GRPs-FW/FL

Plasmid pQE70 with the gene encoding GBP152 and GRP[42, 178] and pLacI were co-transformed into the E.coli tryptophan auxotroph ATCC #27873 for expression of GRPs-FW. Plasmid pQE70 with GBP152 and GRP[42, 178] were transformed into the *E. coli* leucine auxotroph HB101F′ for expression of GRPs-FL. Protein expression was performed using the medium shift method[165]. A single freshly transformed colony was used to inoculate 5 mL of M9 media supplemented with 0.4% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM Thiamine, 0.1 volume of a solution containing 0.01% (w/v) each of 19 amino acids (-Leu/ or -Trp), 40 μg/mL Leu/ or Trp and 100 μg/mL ampicillin. This culture was allowed to grow overnight at 37 ^{0}C, 250 rpm. 500 mL culture containing the same ingredients was inoculated with the overnight grown 5 mL culture. This culture was then grown till the optical density (OD_{600}) was ~0.5- 0.6. The cells were then centrifuged at 10,000 rpm for 10 min, 25 ^{0}C, the supernatant was discarded and the cells were

resuspended in a 0.9% NaCl solution for washing, and this was repeated three times. The cells were then resuspended in 500 mL M9 minimal media which was supplemented with 0.4% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM Thiamine, 0.1 volume of a solution containing 0.01% (w/v) each of 19 amino acids (-Leu/ or -Trp), 100 μg/ml ampicillin, and grown at 37 ^{0}C for 30 min to further deplete leucine. 1 mM Isopropyl β-D-1-thiogalactopyranoside, IPTG was then added with 0.1 mM of 5,5,5-fluoroleucine/ or 5-fluorotryptophan to the culture and was grown overnight at 37 ^{0}C. The cells were harvested by centrifugation and the protein was purified using Ni-NTA resin. The purified protein was dialyzed against 3 changes of 10 mM Hepes, 0.2 mM $CaCl_2$, pH 7.2.

Mass Spectrometry

SDS Page gel pieces of all three proteins with unnatural amino acids were digested with trypsin, and LC-ESI-MS-MS was performed using a ThermoFinnigan LTQ. Resulting MS-MS spectra were searched against proteins in the Swiss-Prot database using the mascot search engine (Matrix Science). The difference in mass of GBP152 and GRPs confirmed the incorporation of the unnatural amino acids.

Circular Dichroism

CD measurements were performed with a Jasco model J-810 spectropolarimeter with a 1 nm bandwidth using a 0.1 cm pathlength cuvette for the far UV scans. The concentration of proteins used for far UV scans and temperature scan were 0.10 mg/mL of GRP-FW, 0.29 mg/mL of GRP1-FW, 0.20 mg/mL of GRP2-FW, 0.17 mg/mL of GRP-FL, 0.18 mg/mL of GRP1-FL, and 0.29 mg/mL of GRP2-FL. The proteins were

dialyzed against a low salt buffer (5 mM phosphate) before CD measurements. The thermal stability of GGBP secondary structure was monitored by heating samples over the range 0 - 99 °C. The mean residue molar ellipticity at 222 nm was recorded at 0.2 °C intervals using a time constant of 8 s.

Labeling GRPs-FL/FW with MDCC (N-[2-(1-maleimidyl(ethyl]-7-(diethylamino)coumarin-3-carboxamide)

The purified protein was incubated with dithiothreitol (DTT) to a final concentration of 2 mM at 4 ^{0}C for 2 h. Excess DTT was removed using YM-10 microcon centrifugal device. The protein was then reacted overnight with 10X MDCC, 4 ^{0}C and in dark. The unreacted MDCC and glucose from the protein was removed by dialyzing against six changes of 10 mM Hepes, 0.2 mM CaCl$_2$, pH 8.0.

Fluorescence study of GRP1-FW/FL and GRP2-FW/FL in solution

The excitation and emission wavelengths of MDCC were 425 nm and 475 nm, respectively. The concentration of labeled proteins used in the fluorescence studies was 1 X 10^{-7} M. The MDCC conjugates were incubated with various glucose concentrations for 1 min. All measurements were done at RT in quartz cuvettes with sample volume of 200 μL.

RESULTS AND DISCUSSION

GBP has a total of 22 leucines and 5 tryptophans. None of the 22 leucines is involved in the formation of bonds with glucose, thus incorporating FL into GBP should not affect the glucose binding ability of the protein. However GRP1 (bigger truncated fragment)[42] has 5 tryptophans and 21 leucines while GRP2 (smaller truncated fragment)[42] has 4 tryptophans and 19 leucines. However, one of all the tryptophans in GBP and its truncated fragments is located within the glucose binding pocket of GBP152.[39] Hence, incorporation of FWs in the protein might affect the binding ability and/or stability of the protein. Also as previously mentioned fluoroleucines have shown to enhance the thermal stability of proteins. Therefore, we hypothesized that global incorporation of FL and FW in GBP152, GRP1 and GRP2 may result in changes in the structure and/or stability of the protein.

In our work we globally incorporated FL/FW into GBP152, a H152C GBP mutant and its two fragments that contains a unique cysteine located in close proximity to the glucose binding site. For the global incorporation of unnatural tryptophans in the GBP152 and GRPs, the pQE70 vector with GBP152 or GRP1 or GRP2 was co-transformed with plasmid placI in *E. coli* tryptophan auxotrophic strain. Plasmid pLacI was transformed in the cells to control the expression of protein. For global incorporation of unnatural leucines in GBP152 and GRPs, the pQE70 vector with GBP152 or GRP1 or GRP2 was transformed into *E. coli* leucine auxotrophic strain. The protein expression was then achieved by medium shift method[165, 178], where the cells are grown in a minimal medium supplemented with salts, glucose and all 20 amino acids then the cells are washed and resuspended in minimal medium supplemented as before but lacking the 20th

amino acid which will be replaced by its unnatural analogue. The cells are starved for the 20th amino acid and then the unnatural amino acid is added to the medium causing the cells to forcefully incorporate the amino acid in the protein. The cells are harvested by centrifugation and then purified using nickel affinity due to the presence of six histidine tag in the plasmid. To confirm the incorporation of unnatural amino acids, the unnatural GRPs were analyzed by LC-ESI-MS-MS. The difference in protein mass obtained confirmed the incorporation of the unnatural amino acids (Table 4.1).

Previously we demonstrated that GBP152 chemically conjugated to fluorescent probes such as N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) not only can bind to glucose, but also causes a modulation of the probe's fluorescence directly proportional to the concentration of glucose.[10] In a similar manner, GBP152/GRPs-FL/FW were covalently attached to MDCC and the effect of glucose on the fluorescence intensity of the probe was studied. The glucose binding activity of GBP152-FW/FL, GRP1-FW/FL and GRP2-FW/FL was then determined in solution by incubating the unnatural proteins with glucose solutions. The dose response curves for the unnatural proteins with FW and FL are shown in Figure 4.2a and 4.2b, respectively. A change in the fluorescence was observed with different concentration of glucose, suggesting that the incorporation of fluorinated tryptophan and leucine did not affect the glucose binding of the unnatural proteins. Apparent K_{ds} were determined to be 5.4 x 10^{-5} M, 2.02 x 10^{-6} M, 4.1 x 10^{-6} M, 7.5 x10^{-4} M, 1.9 x 10^{-4} M and 1.8 x10^{-4} M for GBP152-FL, GBP152-FW GRP1-FW, GRP2-FW, GRP1-FL and GRP2-FL respectively. It is also observed that proteins with unnatural trytophan demonstrate lower detection limits compared to the protein with unnatural leucine.

Table 4.1 Mass spectrometry analysis of trypsin digest of GBP152-FW/FL and GRPs-FW/FL

Protein	Peptide Fragments	Mass in GBP	Mass in GRP	Delta Mass
GBP152-FW	TEQLQLDTAMWDTAQAK	1949.92	1966.91	16.99
	GAADGTNWK	918.42	936.41	17.99
	HWAANQGWDLNK	1438.67	1474.66	35.99
GBP152-FL	DGQIQFVLLK	1160.67	1267.59	106.92
	ESGIIQGDLIAK	1243.69	1297.21	53.52
	ATFDLAK	765.414	818.32	52.91
GRP1-FW	GAADGTNWK	918.42	936.41	17.99
	MDAWLSGPNANK	1303.61	1320.59	16.98
	TEQLQLDTAMWDTAQAK	1949.92	1966.91	16.99
GRP2-FW	HWAANQGWDLNKDGQIQFVLLK	2600.34	2616.30	15.96
	MDAWLSGPNANK	1303.61	1320.59	16.98
	TEQLQLDTAMWDTAQAK	1949.92	1966.91	16.99
GRP1-FL	TEQLQLDTAMWDTAQAK	1949.92	2056.72	106.83
	HWAANQGWDLNKDGQIQFVLLK	2600.34	2742.08	141.74
	NLADGK	617.32	670.55	53.23
GRP2-FL	DGQIQFVLLK	1160.67	1267.49	106.82
	HWAANQGWDLNKDGQIQFVLLK	2600.34	2742.08	141.74
	QNDQIDVLLAK	1256.68	1363.51	106.83
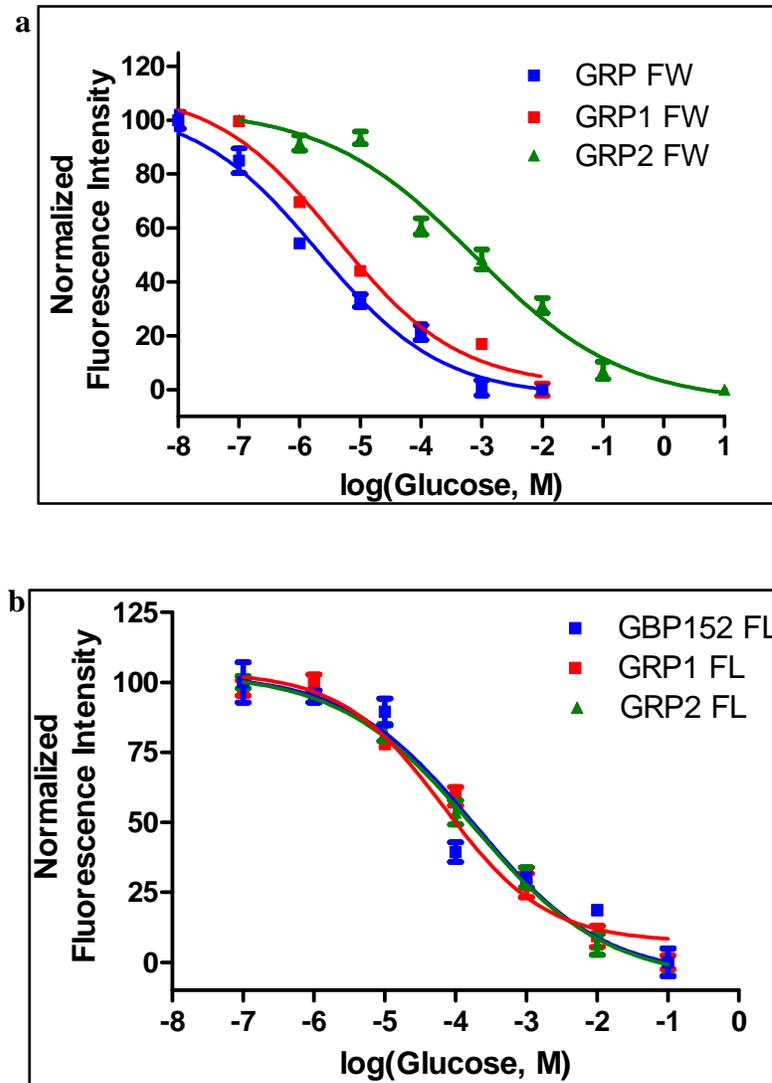
Figure 4.2 Dose response curves for glucose employing proteins with (a) FW (b) FL. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.

The secondary structure of GBP152-FW/FL, GRP1-FW/FL and GRP2-FW/FL were then analyzed by far-UV CD spectroscopy at RT. The CD spectrum of GBP152-FW is similar to the reported CD spectrum of GBP,[36] hence, indicating that the secondary structure of GBP152 is not altered by incorporation of unnatural tryptophans (Figure 4.3a). This is further supported by the apparent K_{ds} of both GBP152 and GBP152-FW in µM range. However, the CD spectrum of GBP152-FL (Figure 4.3b) is different from that of GBP152, as the bands at 222 nm and 208 are shifted and less intense for GBP152-FL. This suggests that the secondary structure of GBP152 is altered by incorporating unnatural leucines and, hence, explains the change in apparent K_d as compared to GBP152. The CD spectrum of GRP1/2-FW (Figure 4.3a) reveals that the incorporation of fluorinated tryptophans in the trunctated proteins, i.e., GRP1/2 alter the secondary structure of protein as compared to the full length GBP152-FW. This can be explained again by both the position and intensity of bands at 222 nm and 208 nm. The band at 222 nm for GRP1/2-FW is less pronounced than the corresponding band for GRP152-FW while the band at 208 nm in GBP152-FW is shifted towards lower wavelength (204 nm) in GRP1/2-FW. Also on comparing the secondary structure of GRP1/2-FW with the natural GRP1/2[42] it is evident that the band around 208 nm as seen in both the natural GRP1/2 is shifted to a lower wavelength of 204 nm in GRP1/2-FW, suggesting a decrease in alpha helical content. However, the CD spectrum of GRP1/GRP2-FL (Figure 4.3b) suggests that the incorporation of fluorinated leucines in GRP1/2 does not alter the secondary structure as compared to GRP1/2, but is altered as compared to GBP152.

The Thermal stability of the GBP152, GBP152-FW/FL, (Figure 4.4a) GRP1/2-FW (Figure 4.4b) and GRP1/2-FL (Figure 4.4c) was determined by temperature-induced
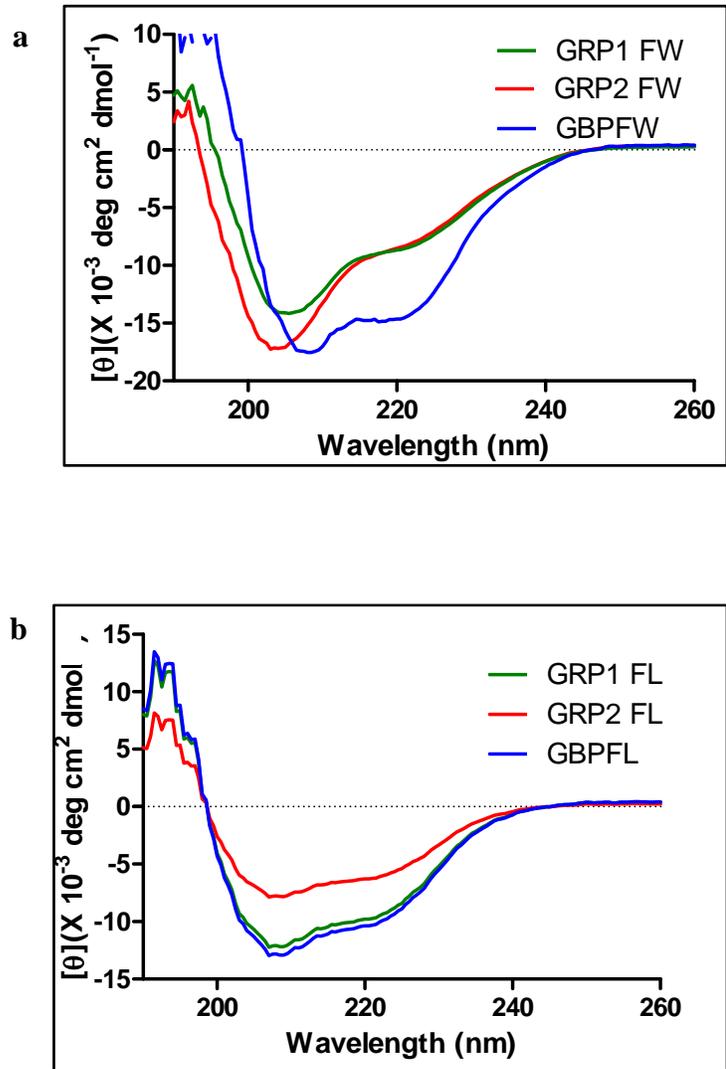
Figure 4.3 Secondary structure of proteins as determined by far-UV Circular Dichroism spectroscopy at room temperature. (a) GBP152-FW and GRP1/2-FW (b) GBP152-FL and GRP1/2-FL.
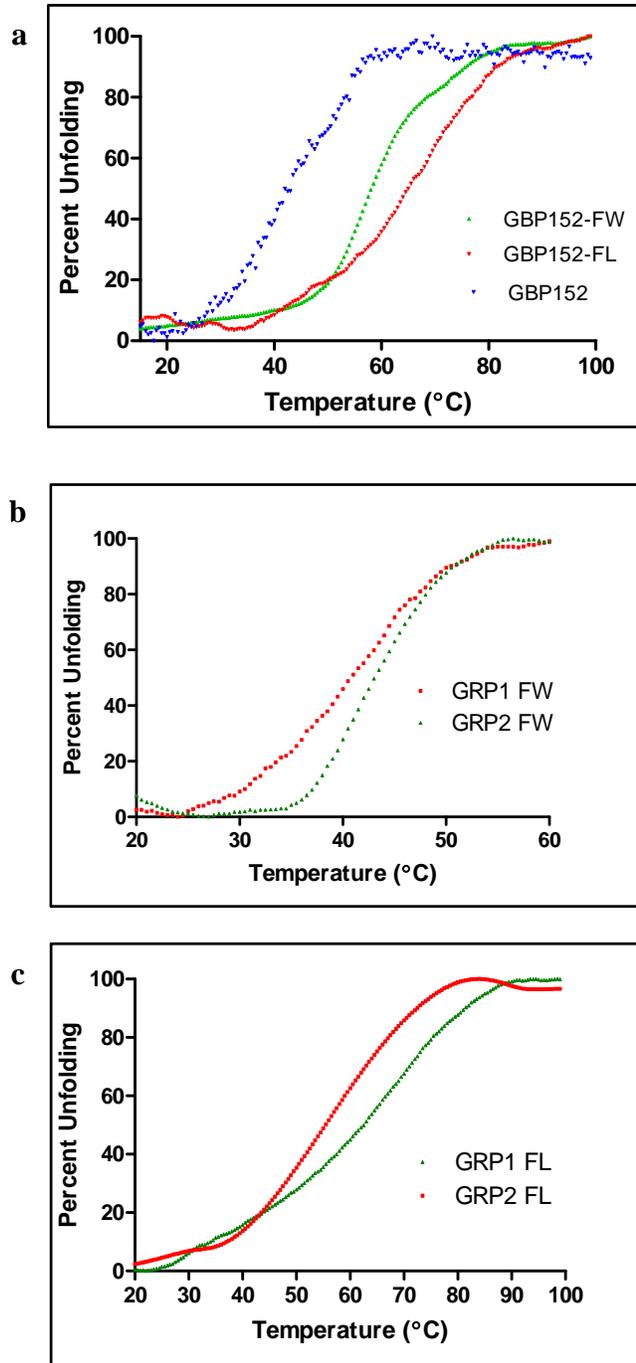
Figure 4.4 Thermal stability as determined by Circular Dichroism spectroscopy at 222 nm. (a) GBP152, GBP152-FW and GBP152-FL (b) GRP1/2-FW (c) GRP1/2-FL.

CD changes at 222 nm. On comparing the thermal stability of the unnatural amino acid incorporated GRPs with the reported natural amino acid containing GRP1/2[42] , the GRP1/2-FW/FL show enhanced thermal stability. Also the thermal stability of GBP152-FW/FL was enhanced as compared to GBP152. The melting temperatures, T_m, were determined to be 47 ^{0}C, 66.1 ^{0}C, 58.9 ^{0}C, 41.0 ^{0}C, 43.1 ^{0}C, 65.3 ^{0}C and 56.5 ^{0}C for GBP152, GBP152-FL, GBP152-FW, GRP1-FW, GRP2-FW, GRP1-FL and GRP2-Fl respectively. Further, incorporation of fluorinated leucines demonstrated a larger increase in melting temperature as compared to fluorinated tryptophans. It is also observed that increase in T_m can be correlated to the number of leucines/tryptophans in the protein. GRP1 has 21 leucines and GRP2 has 19 leucines. GRP2-FL, which has a total of 6 fluorines less than GRP1-FL, has a T_m that is lower by 10 ^{0}C than GRP1-FL. However, GRP1/2-FW have similar Tm as there is a difference of only one fluorine in the structure of the two proteins.

CONCLUSIONS

In summary, we illustrated a method to incorporate unnatural amino acids into glucose recognition proteins to alter their stability and properties. The global incorporation of fluorinated tryptophans and fluorinated leucines in the proteins affect the secondary structure of the proteins when compared to the secondary structure of GBP152 but the ability to bind to glucose is not compromised. More importantly, FL and FW enhance the thermal stability of the sensing proteins. The enhanced thermal stability and altered K_{ds} of these newly prepared GRPs makes them especially suited for long-term

glucose sensing applications in a variety of platforms and devices[179]. Moreover, it paves the way for the design and preparation of other binding proteins with tailor-made characteristics for a variety of biotechnology applications.

CHAPTER FIVE

REAGENTLESS FIBER OPTIC BIOSENSOR FOR THE CONTINUOUS MONITORING OF GLUCOSE

INTRODUCTION

Diabetes affects millions of people worldwide resulting in a series of long term health consequences, including cardiovascular diseases[180-182], neuropathy[183, 184] and blindness.[185, 186] Management of diabetes has initiated an urgent need for the close control of glucose in body fluids. The invention of a glucose self-monitoring device in 1971 revolutionized modern diabetes care by allowing diabetics to live better lives though greater control of their diabetes.[187] In-vivo monitoring of glucose levels, therefore, plays an important part in the management of diabetes. The current gold-standard method for measuring blood glucose is the finger prick method, which involves detection with a glucose biosensor. Hyperglycemia is also related to increased in-hospital mortality and morbidity in patients with cardiovascular diseases.[188, 189] However, studies have indicated that there is a need for monitoring blood glucose levels in not only diabetics but non-diabetic in-hospital patients.[190] Attempts have been made for tight control of blood glucose levels in-hospital patients via point-of-care (POC) devices and have demonstrated a decrease in morbidity, mortality, and other complications for patients in critical care conditions.[191-193] Most of the commercial glucose monitoring devices utilize electrochemical signal transduction based on the enzymatic activity reaction of either glucose oxidase[194-196] or glucose dehydrogenase.[197] However, these devices suffer from a series of drawbacks such as the presence of interferences in the blood, poor control of

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activity due to variation in oxygen levels, and electrode fouling.[198] Another limitation related to poor sensitivity of the glucose sensors in the hypoglycemic range[198] and the somewhat limited stability of the proteins used as the recognition elements.[199] Reports regarding the elevation of blood glucose levels and hence, worsening glycemic control in patients with diabetes under stress due to hypothermia, sepsis, etc. have underscored the need for a more accurate and stable continuous glucose monitoring device.[200]

Dexcom's SEVEN+ and Medtronic's MiniMed Guardian are among the few continuous glucose monitoring devices available in the market. These devices measure subcutaneous glucose electrochemically in interstitial fluid. Yet, they suffer from problems such as substantial sensor drift and poor reliability.[201] Given the above limitations it is not surprising that there is still a demand for glucose biosensors that are stable over time, can perform at temperatures covering all physiological thermic states, and are capable of sensing glucose at hypoglycemic, normal and hyperglycemic ranges.

To that end, the design and development of fluorescence-based glucose sensor is reported. The sensor consisting of the use of a mutant of the Glucose/Galactose Binding Protein, GBP152, or unnatural amino acids incorporated GBP152 or unnatural amino acids incorporated truncated fragments of GBP 152, referred to as Glucose Recognition Proteins (GRPs) as the sensing element, which is covalently attached within an optically transparent acrylamide hydrogel immobilized on the tip of an optical fiber for detection of glucose.

GBP of *Escherichia coli* is a periplasmic binding protein that binds glucose with high affinity (K_D= 0.2 μM)[202] and is an attractive candidate for the development of glucose biosensors.[203, 204] The mature GBP is a 33 kDa[36] ellipsoidal protein and consists

of two distinctly similarly folded globular domains which are connected to each other by three peptide segments. The sugar binding site is located in a cleft formed between the two domains of the protein.[39] The binding of glucose is accompanied by a conformational change that affects the hinge region. In the open form of GBP (absence of glucose), the two domains are far apart and the cleft is exposed to the solvent, while in the closed form, the glucose is engulfed within the cleft.[39] GBP mutants with fluorophores in or near the binding pocket have been successfully employed for the development of reagentless glucose fluorescent biosensors by us and others.[8, 10] GBP 152 was chosen as recognition element in our sensor because it has been previously reported by our group that this mutant when labeled with a fluorophore and in the presence of glucose undergoes a quenching of 30 % in its fluorescence intensity, thus making it ideal for the development of biosensors.

GRPs are obtained by incorporating unnatural amino acids into the structure of GBP and its truncated fragments. In an attempt to design glucose sensing proteins with a minimum structure of GBP required for maintaining its affinity to glucose we have previously truncated GBP into two fragments.[42] These fragments are referred to as GRP1 and GRP2 throughout the manuscript. GRP1 and GRP2 (collectively GRP1/2) have demonstrated affinity to glucose. GRP1/2 have been assumed to bind glucose in a similar manner to GBP. Further labeling GRP1/2 with a fluorescent probe has enabled fluorescence based glucose sensing in glucose solutions. It has been previously demonstrated that unnatural amino acids, 5-Fluorotryptophan (FW) and 5,5,5-Trifluoroleucine (FL), can be incorporated in GBP[178] and GRP1/2 utilizing the global incorporation method. Incorporation of fluorinated amino acids into the structures of

GBP/GRP1/GRP2 have resulted in altered glucose binding affinities and enhanced thermal stability.

The fluorescence based-biosensor was developed by covalent attachment of the fluorophore N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide, MDCC in GBP152/GRPs-FW/FL. Further, the MDCC-labeled protein was immobilized within the hydrogel on the tip of an optical fiber (Figure 5.1). A hydrogel was employed to allow for high loading of the protein, and also to incorporate a diffusion barrier capable of shifting the detection range of GBP/GRPs-FW/FL from the micromolar to the milimolar range, which is necessary for *in vivo* glucose detection in hypoglycemic, normal, and hyperglycemic ranges. The response of the fiber optic glucose biosensor was evaluated in standard glucose solutions, in human serum and in pig blood, by monitoring the changes in fluorescence intensity of the probe. The response of the sensor was also studied at different temperatures, 35.5 ^{0}C – 42 ^{0}C, to reflect physiological situations ranging from hypothermia to hyperthermia.
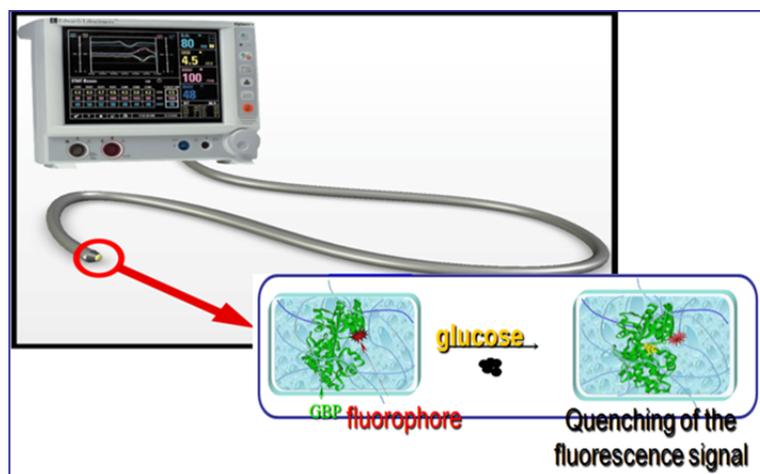
Figure 5.1 Schematic of a fiber optic biosensor for glucose. GBP labeled with a fluorophore is covalently attached to the hydrogel and the hydrogel is immobilized on the tip of the optical fiber connected to a readout device.

EXPERIMENTAL PROCEDURES

Materials

Luria Bertani (LB) medium was purchased from Fischer Scientific (Fair Lawn, NJ). Ampicillin sodium salt, anhydrous calcium chloride, acrylic acid, acrylamide, 2,2-Diethoxyacetaphenone, *N,N′*-Methylenebis(acrylamide), 3-(Trimethoxysilyl)propyl methacrylate (MPTS), tetracycline and human serum were purchased from Sigma-Aldrich (St. Louis, MO). Imidazole was purchased from J.T. Baker, Inv. (Phillipsburg, NJ). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Gold Biotechnology, Inc. (Houstan, TX). The Bradford protein assay kit was purchased from Bio-Rad laboratories, Inc. (Hercules, CA). Sodium Dodecyl Sulfate (SDS) was purchased from Curtin Mathesin Scientific, Inc. (Houstan, TX). pQE70, QIAprep Spin Miniprep kit and Nickel-NitriloTriAcetic acid (Ni-NTA) agarose was purchased from Qiagen (Valencia, CA). Tris glycine SDS Page gels and 7-diethylamino-3-((((2-maleimidyl)ethyl-amino)carbonyl)coumarin (MDCC) and Reagents required for TOPO TA cloning were purchased from Invitrogen (Carlsbad, CA). D-Glucose, anhydrous was purchased from Spectrum (New Brunswick, NJ).) Optical fiber with core diameter of 200 μm was obtained from Ocean Optics Inc. (Dunedin, FL).HEPES was purchased from VWR International (Bridgeport, NJ). All solutions were prepared using deionized reverse osmosis (MILL-Q Water purification System, Millipore, Bedford, MA) water. Swine whole blood was purchased from Innovative Research (Southfield, MI), 1-ethyl-3-(3-dimethylaminopropyk)carbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) was purchased from Thermo Scientific (Waltham, MA). PFU Ultra II fusion HS

polymerase and other reagents required for PCR were purchased from Agilent. T4 DNA ligase was purchased from Promega (Madison, WI).

Apparatus

Cell cultures were grown on orbital shaker from Forma Scientific (Marrietta, OH) and Beckman J2-MI centrifuge (Palo Alto, CA) was used for harvesting the cells. GBP152 was obtained by lysing the cells with 550 sonic dismembrator from Fisher Scientific. Varian Carry Eclipse fluorescence Spectrophotometer was used for fluorescence study of GBP152 in solution. UVGL-58 hand held UV lamp for polymerization of hydrogel was obtained from UVP (Upland, CA). A thermal water circulator from Polyscience (Niles, IL) was used for temperature study. USB4000 Miniature Fiber Optic Spectrometer, obtained from Ocean Optics (Dunedin, Fl), was used for fluorescence measurements of hydrogel.

Construction of plasmid pSDGBP

The polymerase chain reaction (PCR) was performed to introduce a SphI restriction site at the 5' end and a BamHI site at the 3' end of the GBP152 coding sequence. pSD502[10] was used as a template for PCR with PFU Ultra II fusion HS polymerase and primers in a 30-cycle PCR. The primers used were GBPforSph: 5'-GAGGCATGCTTGATACTCGCATTGGTGTAACAATC-3' and GBPrevBam: 5'-CTCGGATCCTTTCTTGCTAAATTCAGCCAGGTTGTC-3'. Cycle conditions were 95 °C x 5 min followed by 30 cycles of 95 °C x 30 s, 55 °C x 30 s, 72 °C x 1 min, 72 °C x 7 min, then Taq polymerase and dNTPs were added to the reaction mixture for TA cloning, followed by 72 °C x 15 min. Reaction was run on TAE 1% agarose gel and the PCR

product was cut out and extracted from the gel. The extracted product was TOPO TA cloned following the method provided with the kit from Invitrogen. TA clones were grown in LB medium and then screened for the insert of the coding sequence for GBP152 by digesting with SphI and BamHI. The digested product was run on TAE 1% agarose gel and then extracted from the gel using QIAprep Spin Miniprep kit. The extracted product was then ligated into SphI and BamHI sites of pQE70 and thus resulting in plasmid pSDGBP. Ligation was carried out overnight at room temperature using T4 DNA ligase. pSDGBP was then transformed into TOP10F' cells using a modified rubidium chloride method.[205]

Expression and Purification of GBP and GRPs-FW/FL

For expression of GBP mutant with Cys at position 152, 10 ml LB broth containing 100 μg/ml ampicillin and 12 μg/mL tetracycline was inoculated with a single *E.coli* colony containing the pQE70 vector with GBP152. The culture was grown overnight (17-18 h) at 37^0C at 250 rpm. Next day 500ml LB broth containing 100 μg/ml ampicillin and 12 μg/ml tetracycline was inoculated with the 10 ml of the overnight culture and was grown at 37 ^{0}C at 250 rpm to an OD_{600} of 0.2. The culture was then grown at room temperature at 250 rpm to an OD_{600} of 0.4. The GBP expression was then induced by adding IPTG to 1mM final concentration. The culture was then allowed to grow for 3 h at room temperature at 250 rpm and the cells were then harvested by centrifugation at 12,000 rpm for 15 min at 4 ^{0}C.

Plasmid pQE70 with GRP[42, 178] and pLacI were co-transformed into the E.coli tryptophan auxotroph ATCC #27873 for expression of GRPs-FW. Plasmid pQE70 with

GRP[42, 178] were transformed into the E.coli leucine auxotroph HB101F′ for expression of GRPs-FL. Protein expression was performed using medium shift method[165]. A single freshly transformed colony was used to inoculate 5 mL of M9 media supplemented with 0.4% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM Thiamine, 0.1 volume of a solution containing 0.01% (w/v) each of 19 amino acids (-Leu/ or -Trp), 40 μg/mL Leu/ or Trp and 100 μg/ml ampicillin. This culture was allowed to grow overnight at 37 ^{0}C, 250 rpm. 500 mL culture containing the same ingredients was inoculated with the overnight grown 5 mL culture. This culture was then grown till the optical density (OD_{600}) was ~0.5- 0.6. The cells were then centrifuged at 10,000 rpm for 10 min, 25 ^{0}C, the supernatant was discarded and the cells were resuspended in a 0.9% NaCl solution for washing, and this was repeated three times. The cells were then resuspended in 500 mL M9 minimal media which was supplemented with 0.4% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM Thiamine, 0.1 volume of a solution containing 0.01% (w/v) each of 19 amino acids (-Leu/ or -Trp), 100 μg/mL ampicillin, and grown at 37 ^{0}C for 30 min to further deplete leucine. 1 mM Isopropyl β-D-1-thiogalactopyranoside, IPTG was then added with 0.1 mM of 5,5,5-fluoroleucine/ or 5-fluorotryptophan to the culture and was grown overnight at 37 ^{0}C.

For the extraction of GBP/GRPs-FW/FL, the cell pellet was resuspended in 30 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and the cytoplasmic GBP was released from the cells by sonication (5 min cycle, 10 sec ON and 10 sec OFF). The sonicated cells were removed by centrifugation at 12,000 rpm for 20

min at 4 ^{0}C and the supernatant was collected. 2ml of Ni-NTA agarose resin were added to the cell lysate, which was then incubated at 4 ^{0}C on a rotor for 1 h. The cell lysate with the resin was poured in a column and the unbound resin was allowed to flow through. The column was then washed with 30 mL of wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0). The protein was eluted by adding the elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0) to the column and 1 ml fractions were collected. The purity of proteins was determined by SDS-PAGE, using 12% acrylamide gels that were stained with Commassie blue. The purified protein was dialyzed against three changes of 10 mM HEPES, 0.2 mM CaCl$_2$, pH 7.0 over 12 h. The purified protein concentration was determined by Bradford assay.

Labeling GBP/GRPs-FW/FL with MDCC

DTT was added to the purified protein solution to a 2 mM final concentration. The resulting solution was incubated at 4 °C to reduce any disulfide linkages that may have formed between two protein molecules. After the reduction was complete DTT was removed from the protein solution by running the sample through Microcon Ultracel YM-10 and resuspending the obtained protein concentrate in 10 mM HEPES, 0.2 mM CaCl$_2$, pH 7.0. The conjugation of the fluorophore to the protein was achieved by mixing 1 equivalents of the protein with 10 equivalents of MDCC. The resulting solution was incubated in dark at 4 ^{0}C overnight. Then the excess fluorophore was removed from the MDCC labeled protein by dialyzing the protein solution against six changes of 10 mM HEPES, pH 7.00, containing 0.2 mM CaCl$_2$, over a period of 24 h.

Fluorescence study of GBP152-MDCC in solution

The excitation and emission wavelengths of MDCC were 425 nm and 475 nm, respectively. The concentration of labeled proteins used in the fluorescence studies was 1 X 10^{-7} M. The MDCC conjugates were incubated with various glucose concentrations for 15 min. All measurements were done at RT in quartz cuvettes in a sample volume of 200 μL.

Conjugation of MDCC-labeled GBP/GRPs-FW/FL with Acrylic acid

For the conjugation reaction, 10 equivalents of acrylic acid were added to a solution containing 2 mM EDC and 5 mM NHS. This mixture was then incubated at room temperature for 2 h with stirring. Then, one equivalent of MDCC labeled proteins in 10 mM HEPES, 0.2 mM $CaCl_2$, pH 7.0 was added to the EDC and NHS treated acrylic acid and incubated in dark at 4 ^{0}C overnight. The acrylic acid conjugated protein was separated from the free acrylic acid, EDC, and NHS by running the sample through a centrifugal filter device (Microcon Ultracel YM-10). The obtained protein was then resuspended in 10 mM HEPES, 0.2 mM $CaCl_2$, pH 8.0.

Preparation of Hydrogel Precursor Solution and Optical Fiber Surface Modification

A hydrogel precursor solution was made from the combination of 8 uL of (1.125 mg acrylamide and 4.125 ug N,N ethylenebisacrylamide in 1 ml), 4 μL 10% glycerol, 9 μL of 1.2 X 10^{-4} M GBP-MDCC-Acrylic acid, and 0.5 μL 2,2-diethoxyacetaphenone. The fiber was cleaned by immersing in 1:1 concentrated HCl: MeOH for 30 min and then rinsed in deionized water and immersed in concentrated H_2SO_4 for 30 min at room

temperature. It was rinsed again by immersing deionized water for 10 min followed by rinsing with ethanol. The fiber was then silanized by immersing in MPTS (3-(Trimethoxysilyl)propyl methacrylate) for 1 h at room temperature and then air dried. 0.3 µL hydrogel precursor solution was deposited on the tip of silanized fiber and polymerized under UV for 30 min. The prepared fibers were stored at 4 ^{0}C till further use.

Optimization of sensor response time

The optical fiber with the immobilized GBP152 hydrogel was immersed in 2 mL of glucose solutions of concentrations ranging from 2 mM to 20 mM in buffer for 30 min. The sensor response i.e. the fluorescence was measured after 1 min, 5 min and 30 min for each glucose solution.

Study of the effect of the length of fiber on sensor response

Two different optical fibers with lengths of 38 cm and 45 cm were used. Hydrogel containing GBP152-MDCC was immobilized on tip of each silanized fiber. The fibers with hydrogel were then dipped in different concentrations of glucose separately and the fluorescence from the sensor was measured.

Sensor response

The response of the fiber based GBP152/GRPs-FW/FL hydrogel sensor was determined by immersing the tip of the hydrogel coated fiber in 2 ml glucose (2 mM – 20 mM) solutions containing 10 mM HEPES and 0.2 mM $CaCl_2$ with pH 8.0. The hydrogel was washed with 2 mL of buffer for 2 min after each concentration of glucose. The

fluorescence intensity of the hydrogel with MDCC labeled proteins at 475 nm, excited by the optical fiber at 425 nm, was recorded by Ocean Optics Spectrometer. Similarly, the sensor response was also determined in human serum and in pig blood, both spiked with different glucose concentrations (2 mM – 20 mM). The reversibility of the sensor was determined by immersing the hydrogel coated fiber in 2 ml glucose solutions (20 mM – 2 mM) as described above. Response of the sensor was also determined at different temperatures (35.5 ^{0}C, 37 ^{0}C, room temperature and 42.5 ^{0}C) by immersing the fiber tip with hydrogel in 2ml glucose solutions in glass vials kept in thermal water cycler used to maintain the temperature. All the experiments were performed in dark.

RESULTS AND DISCUSSION

The glucose biosensor reported herein is based on the use of GBP/GRPs-FW/FL immobilized into a hydrogel network, which in turn is positioned at the tip of an optical fiber. The basis for the response of the glucose biosensor is the conformational changes that GBP/GRPs undergo upon binding to glucose. Therefore, in order to investigate and correlate the changes in conformation of protein with glucose concentrations a signal generating molecule into the system capable of reporting such changes was required to be incorporated into the protein structure. For that, a genetically engineered mutant of wild-type GBP, with a unique Cys at position 152 (GBP152), was used to obtain designer GRPs for selectively attaching an environment sensitive fluorophore, MDCC, into the proteins.[42] It has been previously shown by our group that this mutant when labeled with MDCC, in the presence of glucose presents a 30% decrease in the intensity of the

fluorescence signal.[10] In order to purify the protein using an easy method such as a Ni-affinity column, the GBP152 coding sequence was removed from pSD502 and ligated into vector pQE70 that encodes for a histidine tag at the C-terminus of the protein. For regulating the expression of GBP152, plasmid pSDGBP was transformed into TOP10F' cells. However, for expression of GRPs with unnatural amino acids plasmid pSDGBP was transformed into the respective auxotrophic strain of *E. coli*. The purified proteins were first analyzed by SDS-PAGE and concentration was determined by employing the Bradford assay prior to labeleing of the proteins with MDCC using the thiol chemistry to the Cys in the proteins.

The response of GBP152-MDCC in solution was evaluated by incubating it with different concentrations of glucose solutions in 10 mM HEPES, 0.2 mM $CaCl_2$, pH 8.0 and measuring the fluorescence of the samples. Figure 5.2 shows that the fluorescence of GBP152-MDCC decreases with the increase in concentration of glucose. The response of GRPs-FW/FL is discussed in Chapter 4.

The next step involved crosslinking the fluorophore-labeled GBP152/GRPs-FW/FL within an acrylamide optically transparent hydrogel. This hydrogel not only allows for the loading of larger amounts of protein, but it also serves as a diffusion barrier. Furthermore, incorporation of the proteins in a hydrogel allows for the detection range of glucose in physiologically relevant milimolar range. MDCC-labeled proteins were conjugated to acrylic acid by employing well-established protocols of EDC/ NHS mediated activation of amines in amino acids to label the lysines residues in the proteins with acrylic acid. This reaction results in the formation of GBP152/GRPs-FW/FL-acrylic acid conjugates, thereby, functionalizing GBP152/GRPs-FW/FL with many allyl groups,
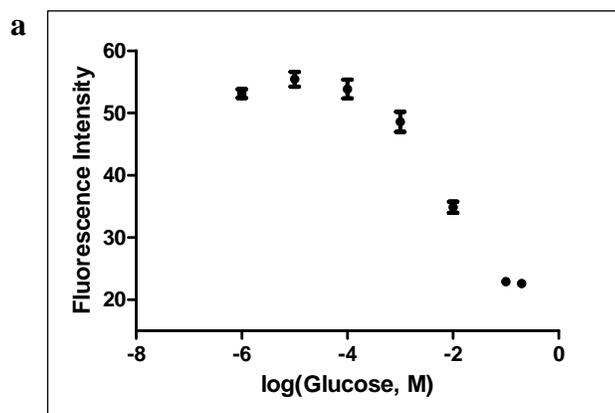
Figure 5.2 Fluorescence study of GBP152-MDCC in presence of glucose. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.

which can be covalently attached within the hydrogel. Although there are 29 Lys in the sequence of glucose binding protein (GBP) eight are partially buried (K11, 92, 104, 147, 189, 227, 270, and 289) and an additional 6 form ion pairs (salt bridges) with acidic residues, including K113 with D111; K191 with D179; K79 with D53; K125 with D299; K169 with D121, K263 with D267 (Figure 5.3a). All these interactions would hinder their accessibility to labeling. Therefore only the more flexible and exposed lysines will be readily labeled with acrylic acid. This accounts for the GBP/GRPs to retain the glucose binding ability.

Furthermore, the optical fiber was silanized with MPTS, which also results in functionalizion of the fiber with allyl groups. This was followed by polymerization between the protein-acrylic acid, with acrylamide (forming the polymer backbone), using bisacrylamide as the crosslinking agent, and 2,2-Diethoxyacetaphenone as the initiator. The resulting polymer crosslinks to the surface of the fiber through the allyl groups when it is placed on the silanized fiber and subjected to UV light to obtain covalent immobilization of the hydrogel on the tip of the silanized fiber (Figure 5.3b).

The MDCC-labeled protein covalently immobilized within the hydrogel matrix demonstrated its capability to emit fluorescence at 475 nm after excitation of the MDCC fluorophore at 425 nm (Figure 5.4). To ensure that the protein is covalently bound within the hydrogel and does not leach into the solution once the tip of the optical fiber is immersed in buffer, the fluorescence of the GBP152 hydrogel in buffer as well as the fluorescence of the buffer (in which the hydrogel is immersed) was measured every 10 min. Table 1 shows that both, the fluorescence of the hydrogel and that of the buffer,
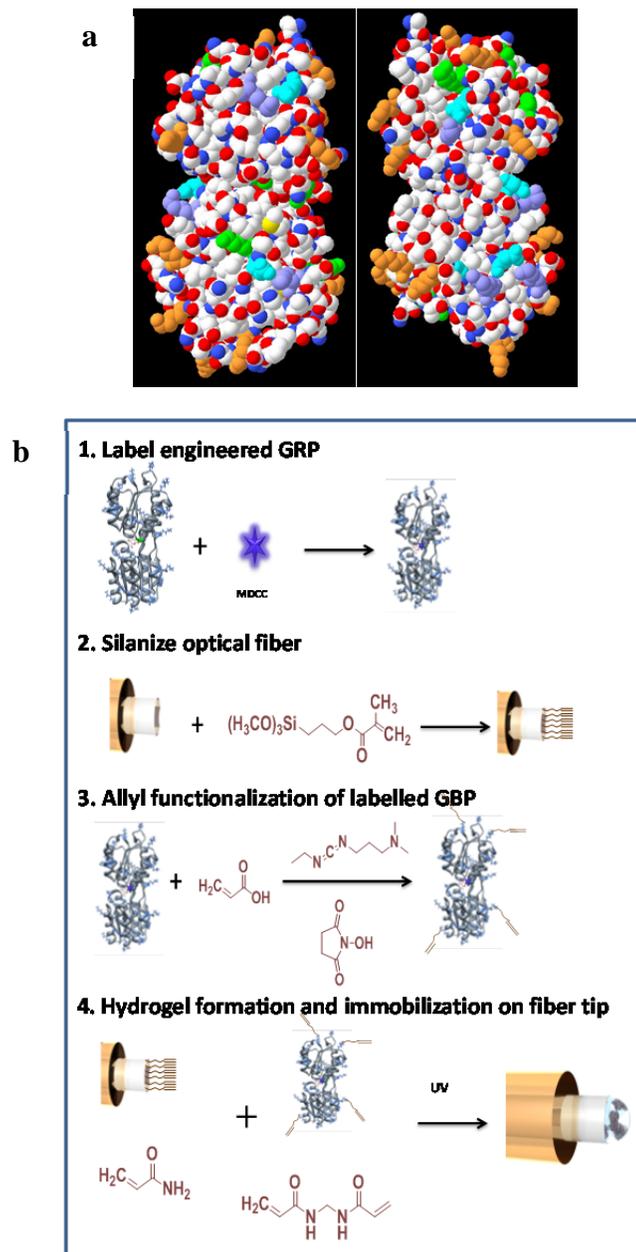
Figure 5.3 (a) Space filled presentation of GBP. Residues other than lysine are colored as blue for nitrogen, white for carbon and red for oxygen. The partially buried lysines are green, the ion-pair forming ones are purple (with their acidic residue partner colored in cyan), and the exposed ones are orange. (b) Schematic of the steps involved in the immobilization of hydrogel containing covalently attached fluorescent GBP/GRPs.
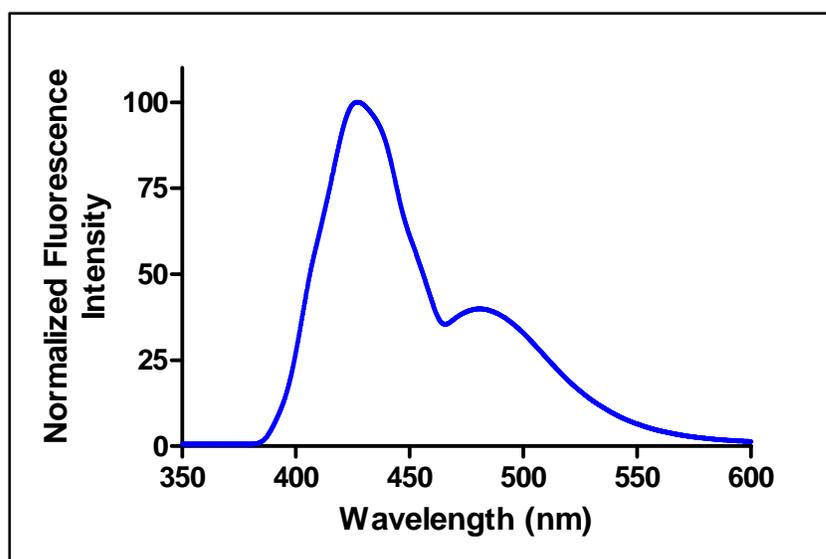
Figure 5.4 Excitation and emission wavelengths (425 nm and 475 nm respectively) of MDCC labeled GBP152 immobilized on the tip of a fiber in an optically transparent hydrogel.

Table 5.1 Leaching studies to determine the covalently bound fluorophore labeled GBP152 within the acrylamide hydrogel.

Time (min)	Fluorescence of Buffer	Fluorescence of Hydrogel
0	19.22	583.64
10	19.32	587.18
20	18.64	583.99
40	19.66	589.65
60	20.17	588.08
80	19.07	587.36
100	18.58	588.41

does not change over a period of 100 min. This confirms that the fluorophore-labeled protein is covalently bound and contained within the hydrogel. No leaching of the fluorophore bound-protein is observed.

For the optimization of response time of the sensor, the sensor was immersed in buffer solutions containing glucose in mM concentrations and the fluorescence change recorded over a period of 30 min. The response time for the sensor was determined to be 1 min, as the observed change in the fluorescence intensity in the presence of glucose was completed within 1 min (Figure 5.5)

The reversibility of the sensor was determined by measuring the change in fluorescence when the sensor is immersed in buffer with increasing glucose concentrations (2 mM -20 mM) followed by decreasing glucose concentrations (20 mM – 2 mM). Figure 5.6 shows that the fluorescence signal decreased with the increase in glucose concentration to almost the same extent as the increase in signal with the decrease in glucose concentration. Change in the fluorescence intensity observed when going from low to high glucose concentrations is the same as the change in fluorescence intensity observed from high to low glucose concentrations. While the signal generated by the biosensor did not show 100% reversibility, still it returns to the baseline completely. This can be explained due to the increase in the glucose concentration within the buffer upon release of glucose from the hydrogel in going from high to low glucose concentrations.

The effect of the length of optical fiber on sensor response was studied by immobilizing the GBP152 hydrogel precursor solution on two different optical fibers
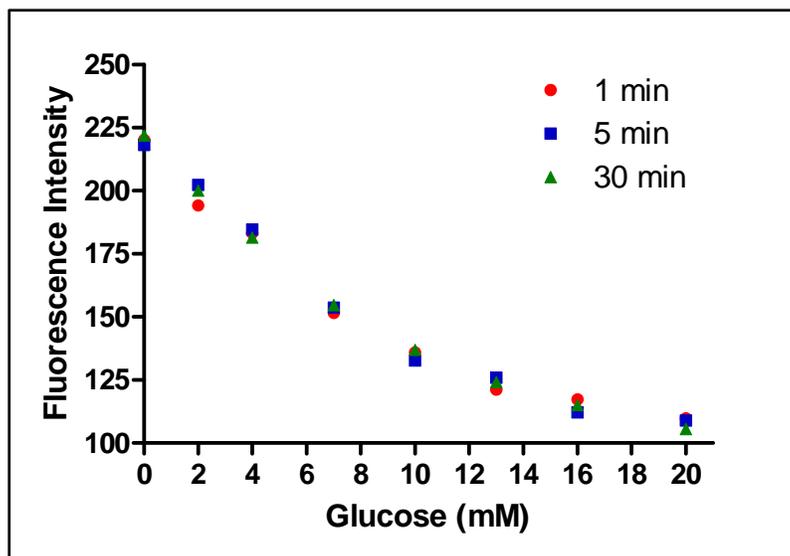
Figure 5.5 Sensor response at different time intervals. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.
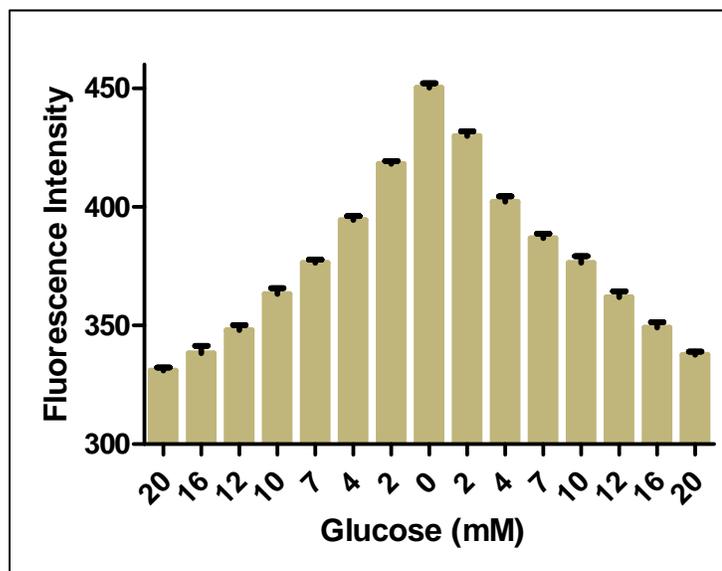
Figure 5.6 Sensor reversibility. Data are the average of ± one standard deviation (n=3).

Relative standard deviations at all concentrations are less than 10%.

with different lengths (Figure 5.7). The sensor response for each fiber was then determined by measuring the fluorescence intensity of the covalently immobilized protein hydrogel when dipping the hydrogel in glucose solutions. It was observed that the shorter fiber (38 cm) showed higher fluorescence intensity at each concentration of glucose than the longer fiber (45 cm). This can be explained by the loss of light as it travels through the fiber.

The sensor response was also studied over a period of three days. After three days it was observed that the mechanical integrity of the hydrogel was starting to fade. For this, a GBP hydrogel was immobilized onto the tip of a silanized fiber and its response was measured during three consecutive days as described previously in different glucose solutions. The fiber was stored at 4 °C in buffer and in the dark after measuring the response each day at 37 °C. The results (Figure 5.8) show that the sensor response was not affected by storage in solution. The immobilized protein was still active and its binding to glucose was not affected by storage and time for at least three days.

Response of the sensor was studied in buffer by immersing the silanized fiber with fluorophore labeled GBP152/GRPs-FW/FL hydrogel immobilized on the tip, in glucose solutions of concentrations within mM ranges. As shown in Figure 5.9a, 5.9b, and 5.9c, the fluorescence intensity decreases with the increase in glucose concentrations. The binding of glucose to GBP152/GRPs-FW/FL causes a change in the microenvironment of the glucose binding site that is manifested by an alteration in the fluorescence properties of the fluorophore in a directly proportional manner to the concentration of glucose present in the sample. The development of the hydrogel
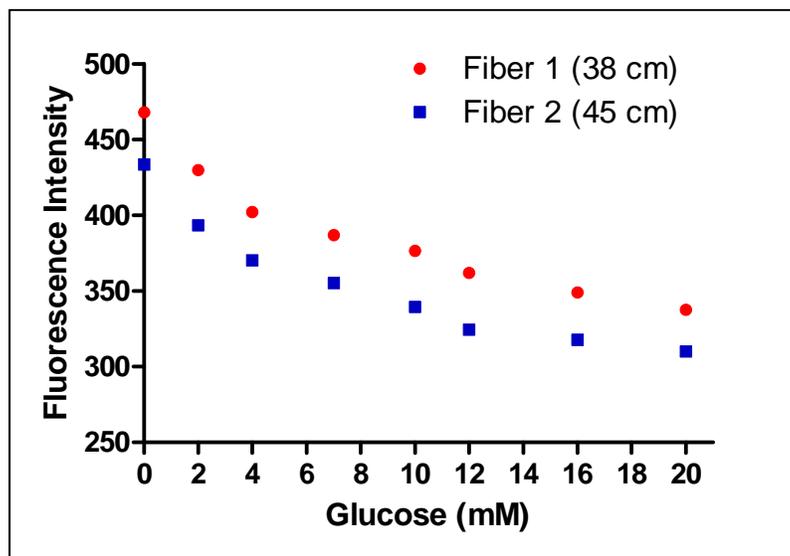
Figure 5.7 Response of two different sensors with GBP152 immobilized within the hydrogel. The sensors differ in the length if optical fiber used. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.
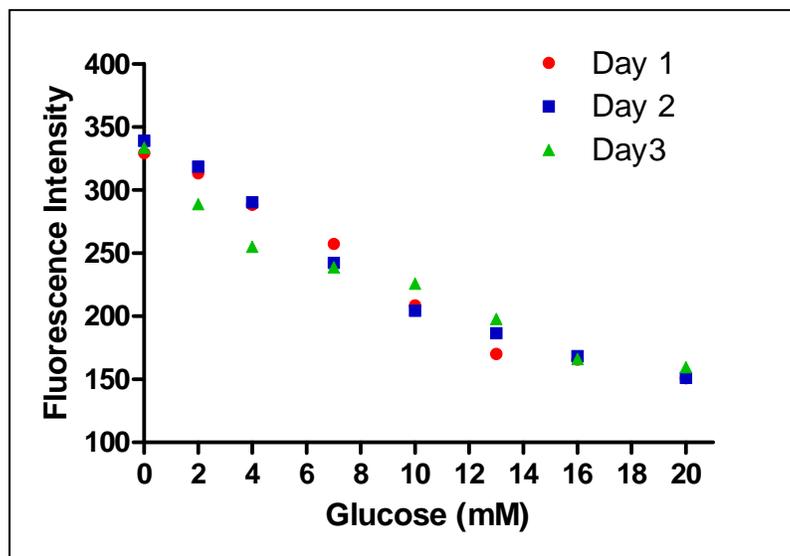
Figure 5.8 Response of hydrogel based fiber optic sensor with covalently immobilized GBP152 on three consecutive days. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.
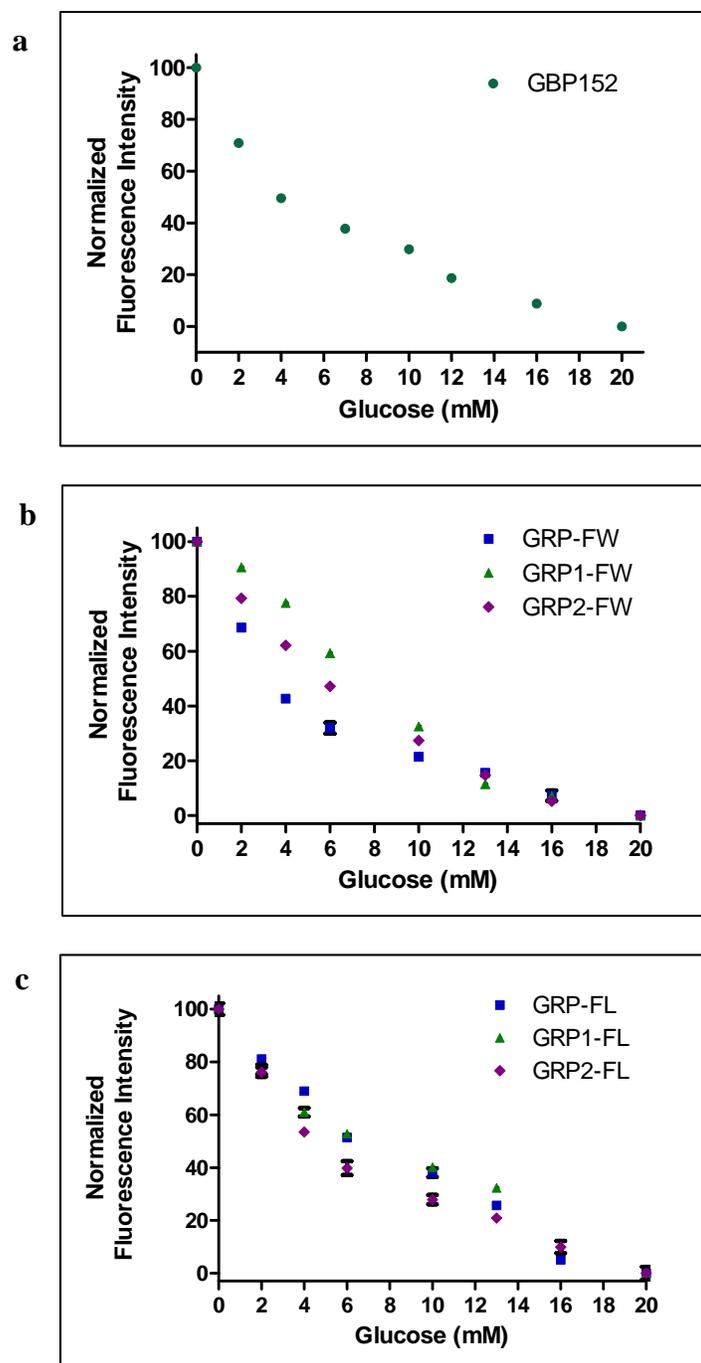
**Figure 5.9** Fluorescence study of fiber optic sensor in physiologically relevant glucose concentrations (a) GBP152 (b) GRPs-FW (c) GRPs-FL. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.

biosensor allows the detection of glucose in the desired physiological mM range by incorporation of a diffusion barrier provided by the hydrogel matrix.

In order to study the performance of the GBP152/GRPs-FW/FL sensor in physiological fluids, the sensor response was studied in human serum (Figure 5.10a and 5.10b) and pig blood (5.11a and 5.11b). A quenching in the fluorescence signal was observed with the increase in glucose concentration, both in spiked human serum and pig blood for all the sensors. Since the initial glucose concentration in pig blood and human serum was 1.6 mM and 6.5 mM, respectively as determined by Free Style Lite (Abott) blood glucose monitoring system, human serum and pig blood were spiked to reach the glucose level upto 20 mM. The performance for GBP152/GRPs-FW/FL sensor was also evaluated by measuring its response at different temperatures such as RT, 37 °C and 42.5 °C (Figure 5.12a-d and Figure 5.13a-c). The results obtained are those that we would expect given the demonstrated thermal stability of the GBP152/GRPs-FW/FL. Since the GBP152, GRP1-FW, and GRP2-FW have Tm at 47 ^{0}C, 41.0 ^{0}C, and 43.1 ^{0}C respectively, the sensors incorporating these proteins showed very little change in fluorescence in the presence of different glucose concentrations at 42.5 ^{0}C. However, GRP-FW[178], GRP-FL[178], GRP1-FL and GRP2-FL with Tm at 58.9 ^{0}C, 66.1 ^{0}C, 65.3 ^{0}C, and 56.5 ^{0}C respectively showed fluorescence quenching at both 37 ^{0}C and 42.5^{0}C. Thus, GRP-FW and GRPs-FL retain glucose binding activity at higher temperatures. Thus, the sensors incorporating designer proteins with enhanced thermal stability can be utilized for the monitoring of glucose within a range of temperatures including those in hypothermic and hyperthermic ranges.
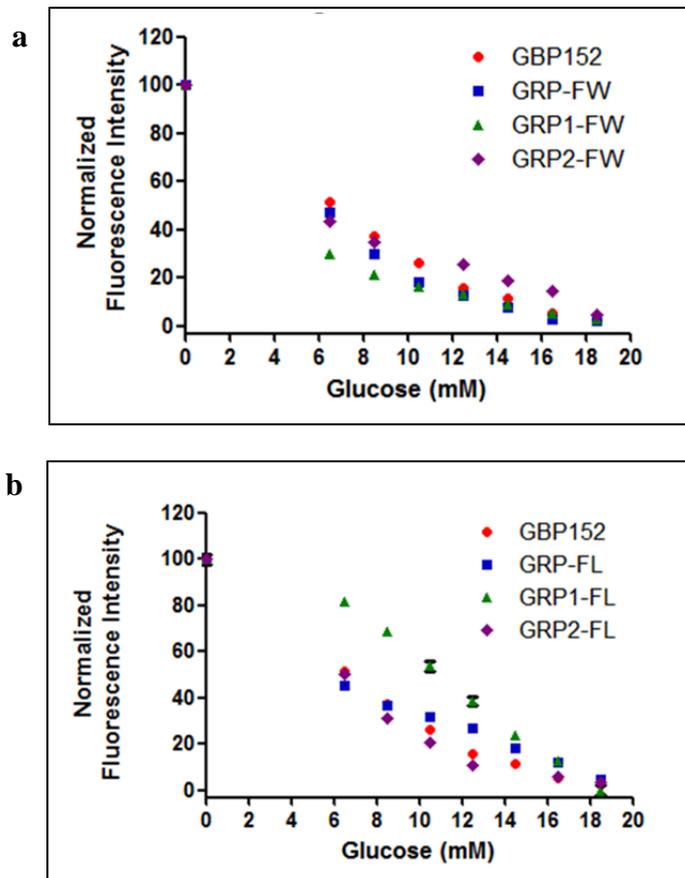
Figure 5.10 Fluorescence study of fiber optic sensor in spiked human serum (a) GRPs-FW (b) GRPs-FL. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.
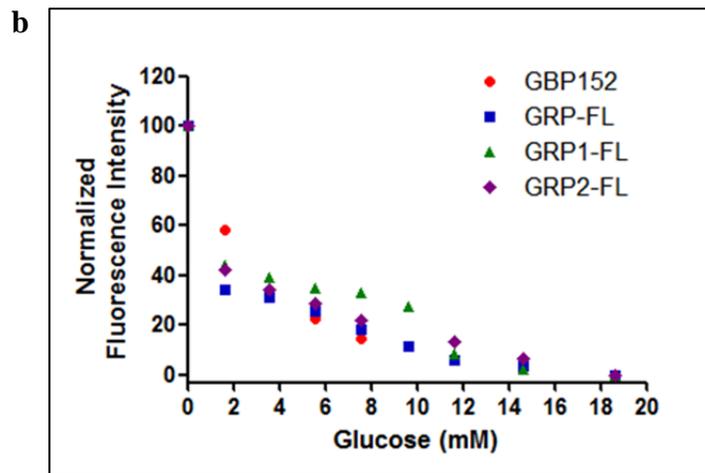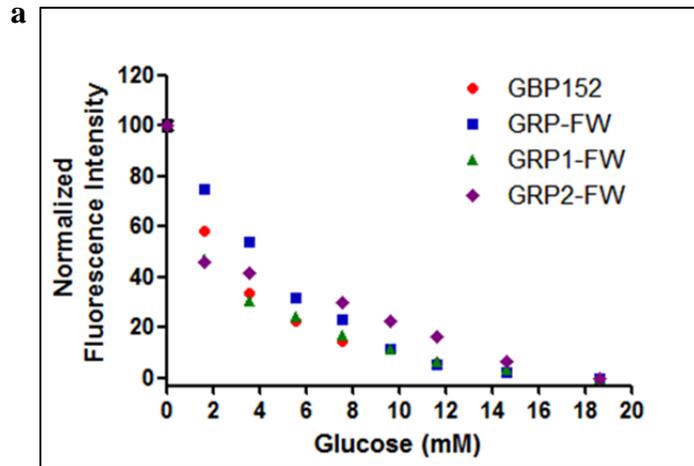
Figure 5.11 Fluorescence study of fiber optic sensor in spiked pig blood (a) GRPs-FW

(b) GRPs-FL. Data are the average of ± one standard deviation (n=3). Relative standard

deviations at all concentrations are less than 10%.
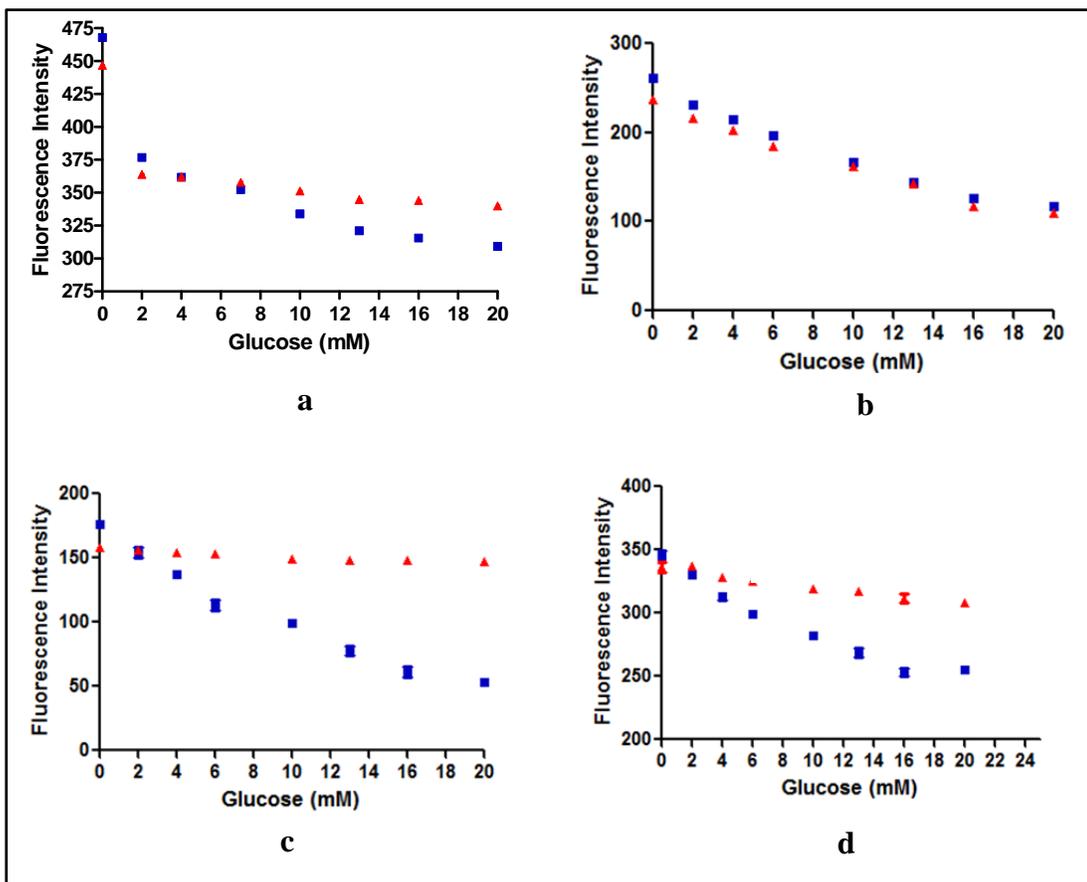
**Figure 5.12** Temperature study of GBP142 and GBP-FW sensor (a) GBP152 (b) GRP-FW (c) GRP1-FW (d) GRP2-FW. Symbols represent ■ 35.5 °C, and ▲ 42.5 °C. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.
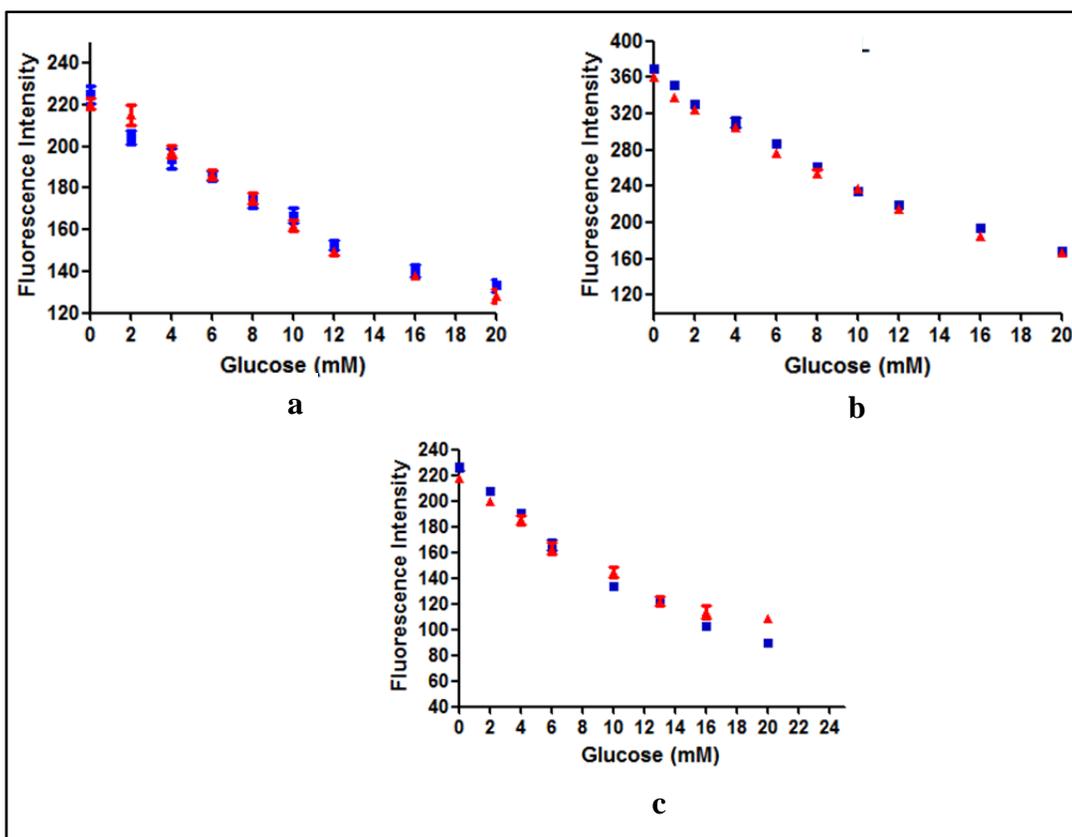
**Figure 5.13** Temperature study of GRPs-FL sensor (a) GRP-FL (b) GRP1-FL (c) GRP2-FL. Symbols represent ■ 35.5 °C, and ▲ 42.5 °C. Data are the average of ± one standard deviation (n=3). Relative standard deviations at all concentrations are less than 10%.

CONCLUSIONS

The existing need for continuous glucose monitoring in diabetics as well as in-hospital patients instigated the development of our fiber optic-based glucose monitoring systems. As previously mentioned, the currently available glucose sensing devices have a number of limitations. In addition to the devices described in this chapter others such as the,"GluCath", an intravascular continuous monitor product from GluMetrics, Inc., is still in the developmental stages.[206] GluCath is based on fluorescent derivatives of boronates. Although the product claims to be sensitive in hypoglycemic ranges, it suffers from some major limitations. These limitations arise from of the fact that boronic acid derivatives based-glucose sensors are known to be pH sensitive[74] and to bind to glycosylated proteins.[207, 208] Thus, these impose a challenge when using boronates to measure glucose in the clinic under physiological conditions.

In this research fiber optic-based reagentless fluorescence biosensor for glucose, comprising of a miniaturized hydrogel that incorporates glucose binding protein,GBP152 or designer glucose recognition proteins, GRPs-FW/FL were developed. Immobilizing the protein within the hydrogel network provides protection to the protein and worsens the detection limit to our advantage. Thus, enables the sensing of glucose in physiologically relevant mM ranges. The fiber optic glucose biosensors demonstrated a fast response time, ~ 1 min, at different physiological temperatures ranging from 35.5 °C to 42.5 °C. The biosensors are highly selective and sensitive to glucose. Moreover, the biosensors were capable of glucose detection in different samples, including human serum and pig blood, without any pretreatment of the sample. Thus, demonstrating its

sensitivity at physiological pH and its potential use in the monitoring of glucose as it overcomes the limitations of the existing glucose sensing systems.

CHAPTER SIX

CONCLUSIONS AND FUTURE PERSPECTIVES

Protein Engineering is a valuable tool for manipulation of protein structure and hence, for better understanding of protein structure and function. Traditional design methods in protein engineering are based, among others, on direct evolution or rational design. In recent years, computational protein design methods have provided further insights into protein structure. Engineered proteins have found application in the development of sensing devices. Fluorescence-based sensing is a powerful tool utilized for the development of fast, reagentless, and non-invasive detection of analytes. Proteins have been engineered to fluoresce by either fusion of the non-fluorescing proteins with fluorescent proteins, conjugation of fluorescent molecules or incorporation of unnatural fluorescent amino acids. In this work, we describe the development of designer proteins and their utilization for the development of fluorescence-based biosensors for glucose and universal biosensing systems for several biomolecules of interest.

Chapter two and three describes the development of universal biosensing systems for the detection of biomolecules of interest. The biosensing system utilizes modified monoclonal antibodies. The non-canonical site in the variable region of antibodies near the antigen-binding site is modified and utilized for signal transduction. The non-canonical site has been reported to covalently bind to nucleotides with azide functionality. Docking to this non-canonical site does not alter the antigen-binding ability

of the antibody. In an effort towards design of antibodies capable of sensing molecules for target analytes, the non-canonical site in the monoclonal antibody was covalently conjugated to a fluorophore labeled azido-nucleotide that acts as the reporter molecule of the biosensing element. This modification at the non-canonical site does not affect the antigen binding, which was demonstrated by testing the biosensing system for two different analytes. The first analyte employed in the proof of concept work was Interleukin 6. Interleukin 6 is a pro- and anti-inflammatory cytokine, whose concentrations is elevated in several disease states, including cardiovascular diseases. The second analyte of interest chosen for our work is Osteonectin, a salivary bone marker. The biosensing system thus developed is universal as it can be utilized for the detection of a variety of analytes depending upon the availability of monoclonal antibody against the analyte of interest.

Future work with respect to the work described in Chapters Two and Three should include understanding the mechanism of response of the fluorescently labeled antibody when exposed to its corresponding ligand analyte. Current studies showed a lag time in generating a response of the antibody when exposure to its ligand. This could be due to a change in conformation of the antibody upon binding the ligand, or simply due to the time needed for difussion of the ligand into the binding pocket of the antibody. To elucidate this mechanism, a time study of the response generated by a series of concentrations (lower than that employed in the study) of fluorescently labeled antibody to its corresponding antigen should be undertaken. This would provide an answer on whether the lag time is due to diffusion or hint toward a delayed conformational change. Future work can be further extended by developing detection systems utilizing the above

mentioned strategy for other biomolecules of interest. Some examples include cortisol, osteocalcin and melatonin. In our work we utilized ATP-modifed probes for conjugation of the fluorophore to the non-canonical site of the antibody. Other nucleotide probes wherein the fluorophore is conjugated to the probe not at the γ-phosphate of ATP, but rather covalently linked to the oxidized ribose can be utilized for labeling the antibody unconventional site. The effect of conjugating the fluorophore at this position might produce a fluorescence signal of similar or different intensity as in previous design and thus, provide a better understanding of the orientation of fluorophore within the non-canonical nucleoside binding site. The effect of antigen-binding to the antibody can be then studied using these fluorescent probes. Further, it has been reported that the non-canonical site has affinity for heterocyclic ring structures and, therefore, the use of other nucleotides, nucleosides and amino acids such as tryptophan should be explored as probes for the covalent attachment of the reporter fluorophore molecule. Also several other fluorophores can be conjugated to the nucleotide probes, allowing for a wider range of emission wavelengths of the reporter molecule.

Chapter four describes the global incorporation of unnatural tryptophan and unnatural leucine into the amino acid sequence of GBP of *E coli* and its truncated fragments. 5-Fluorotryptophan and 5,5,5-trifluoroleucines were incorporated separately in the protein by using a medium shift method wherein the bacteria is first grown in a medium enriched with all 20 amino acids, and then transferred to a medium lacking the 20th amino acid, which is then replaced by its unnatural analogue. By utilizing this method we have been successful in replacing all the natural tryptophans or leucines by their unnatural analogues in the Glucose/Galactose binding protein (GBP) and its

truncated fragments (GRPs). The effect of incorporation of these unnatural amino acids on the secondary structure, glucose binding activity, and thermal stability of the proteins was studied. Circular dichroism spectroscopy in the UV region at room temperature demonstrated that the incorporation of 5-Fluorotryptophans in full length GBP does not affect the secondary structure of GBP. However, the incorporation of fluoroleucines in GBP does alter the secondary structure. Incorporation of either fluorotryptophans or fluoroleucines in truncated GBP fragments does affect the secondary structure of the proteins. This change in the secondary structure does not prevent the GRPs from binding to glucose. However, their glucose binding ability was altered as can be inferred from the apparent K_{ds} of these unnatural proteins (GRPs) compared to natural GBP. The glucose binding ability of these unnatural proteins was determined by labeling the proteins with an environment sensitive fluorophore, MDCC, and generating dose-response curves for glucose and other sugars. All proteins utilized for incorporation of unnatural amino acids contained a unique cysteine that was conjugated to MDCC using the sulfhydryl group of cysteine. Further, thermal stability of the unnatural proteins was determined by far-UV circular dichroism spectroscopy. Incorporation of fluorinated tryptophans or leucines in the proteins demonstrated an increase in the thermal stability of the proteins.

The work described in Chapter four can be further extended by incorporating other unnatural amino acids that would confer these proteins with special properties and functionalities without diminishing their glucose binding capability. Some examples of unnatural amino acids that can be incorporated in the future include benzoylphenylalanine, p-azidophenylalanine and azidonorleucine, which can confer a photoreactive functionality to the protein that can be further harnessed for selective

modification of proteins for sensing purposes. Another example of unnatural amino acid that can be incorporated in these proteins is 2-Ferrocenylglycine, an electroactive non-natural amino. The incorporation of an electroactive amino acid will allow for the further development of electrochemical sensor for glucose utilizing these unnatural electroactive proteins.

In this work, the proteins were labeled with a fluorophore for the development of optical sensors. A future improvement to this work would be the incorporation of a fluorescent amino acid within the structure of the proteins, which would result in genetically encoded fluorescent proteins. An example of such an amino acid is 2-Amino-3-(7-methoxy-2-oxo-2H-chromen-4-yl)propanoic acid, an alanine analogue and a fluorotryptophan. Incorporation of these unnatural amino acids would confer inherent fluorescence to the proteins and, hence, these can be directly utilized for the development of optical sensors.

Chapters five describe the development of fiber optic-based glucose biosensors, wherein, the fluorophore-conjugated glucose recognition element, GBP or GRPs, was covalently immobilized within an optically transparent acrylamide hydrogel. GBP, as well as the unnatural amino acids incorporated GRPs, have a high affinity for glucose with K_{ds} in the 10^{-6} M to 10^{-5} M range. In an effort to develop a glucose biosensor that can detect glucose in physiologically relevant mM concentrations, the fluorescently labeled GBP/GRPs were encaptured within a hydrogel matrix. The Hydrogel matrix serves several purposes as it provides for a hydrophilic, protein friendly environment, allows for high loading of the protein, and serves as a diffusion barrier capable of shifting the detection range of the proteins to the desired mM range. In our

work, the hydrogel was further immobilized on the tip of an optical fiber. The sensor response demonstrated reversibility at low-to-high glucose concentration ranges and viceversa. The ruggedness of the sensor was demonstrated by evaluating the sensor response during three consecutive days. Further the sensor exhibited acceptable response in body fluids such as blood and serum and at physiologically important temperatures. Thus, the sensor performance suggests its potential use for use for *in vivo* glucose monitoring.

Future work for improvement of the biosensing systems described in Chapters five could be accomplished by further enclosing the hydrogel within a dialysis membrane and attached onto the tip of the optical fiber. The dialysis membrane would help to retain the hydrogel integrity over longer periods of storage and use. In order to introduce an internal reference within the fiber optic sensor to eliminate background noise due to the variations in intensity of light, another fluorophore that is not sensitive to the environment can be labeled to the protein at a position that does not interfere glucose binding. Ratiometeric study of emissions of environment sensitive and non-environment sensitive fluorophores can then be performed. This single optical fiber optic based sensor can be used as a catheter for *in vivo* applications as such. However, a dual lumen catheter would extend its utility for in vivo applications, wherein, one lumen would contain the sensor, while another would contain a channel through which blood may be drawn, such that glucose readings from the same blood sample can be analyzed and compared by both the catheter and traditional methods. Further incorporation of more than one hydrogel with the glucose sensing element on different fibers bundled together can be utilized for extending the life of the sensor, as after the lifetime of one sensor has expired other

sensor could be used for glucose recognition in blood. Study of the functioning of the catheters in animal study would enable to determine the performance of the sensor *in vivo*.

In conclusion, antibody-based sensing system for universal detection of analytes of interest was successfully developed by labeling an inherent non-canonical nucleoside binding site with a fluorophore conjugated nucleotide probe. Docking the non-canonical site did not affect the antigen binding. The developed sensing system was demonstrated to be very selective due to the use of monoclonal antibodies. Successful development of glucose recognition proteins with enhanced thermal stability by incorporation of unnatural amino acids in to the structure of GBP and its truncated fragments has been reported. Further, application of the unnatural amino acid incorporated proteins for the development of fiber optic based biosensor for *in vivo* glucose monitoring has also been demonstrated.

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VITA

Smita Joel was born on May 23, 1978 in Jhansi, India. After graduating from high school in India, she earned her Bachelor of Science (B. S.) degree in chemistry in 1999 from Isabella Thoburn College, Lucknow University, India. She then acquired a Bachelor of Education (B. Ed.) degree from Lucknow Christian Training College, Lucknow University, India in the year 2000. She received her Master of Science (M. S.) degree in organic chemistry from Lucknow Christian College, Lucknow University, India in 2002. In Fall 2004, she started the graduate program in the Department of Chemistry at University of Kentucky (UK) in Lexington, KY. In Fall 2005, she joined the research group of Dr. Sylvia Daunert at UK and worked on the development of biosensing system for molecular diagnostics and biosensors using binding proteins. She was awarded the Research Challenge Trust Fund Fellowship and Gill Fellowship during her graduate studies at the University of Kentucky, Lexington, KY.

PATENTS

1. Daunert, Sylvia; Bachas, Leonidas G.; Haley, Boyd; **Joel, Smita**; Moschou, Elizabeth; Turner, Kendrick; Rowe, Laura; Wang, Ping; Siegrist, Jonathan P.; Madou, Marc J. *Fiber-optic device including catheter and biosensor containing glucose-binding protein for continuous in vivo detection of glucose.* PCT Int. Appl. (2009), 58pp. WO 2009021039.

2. Daunert, Sylvia; Turner, Kendrick; **Joel, Smita**; Rowe, Laura. *Stabilized variants of bacterial glucose-binding proteins for use in biosensors for monitoring of blood glucose.* PCT Int. Appl. (2009), 53pp. WO 2009021052.

3. Daunert, Sylvia; Bachas, Leonidas G.; Haley, Boyd; **Joel, Smita**. *Semi-synthetic antibodies as recognition elements containing purine molecular and fluorophore bound to purine.* PCT Int. Appl. (2009), 47pp. WO 2009021026.

PUBLICATIONS

1. J. Siegrist, T. Kazarian, C. Ensor, **S. Joel**, M. Madou, P. Wang, and S. Daunert, **"**Continuous glucose sensor using novel genetically engineered binding polypeptides towards in vivo applications." Sensors and Actuators, B: Chemical (2010), 149(1), 51-58.

2. **S. Joel,** A. Bhattacharya, B. Haley, L. G. Bachas, and S. Daunert, "Biosensors Based on Site-Specific Labeling of Antibodies", to be submitted, 2011.

3. **S. Joel,** T. Head and S. Daunert. "Designer Sensing Proteins via Global Incorporation of Unnatural Amino Acids", submitted, 2011.

4. **S. Joel,** K. Turner, and S. Daunert, "Toward Designer Glucose Recognition Proteins: Preparation of Semi-Synthetic Unnatural Glucose Recognition Proteins", to be submitted 2011.

5. **S. Joel**, L. G. Bachas and S. Daunert, "Reagentless Fiber Optic Biosensors for the Continuous Monitoring of Glucose ", in preparation, 2011.

CONFERENCE PRESENTATIONS

1. Poster presentation at the 238th ACS National Meeting August 16-20, 2009, Washington, DC, United States. "Biosensors based on site-specific labeling of antibodies." **Smita Joel**, Anjan K. Bhattacharyya, Boyd Haley, Leonidas G. Bachas, and Sylvia Daunert.

2. Oral presentation at Pittcon March 11, 2009, Chicago, IL, United States. "Development of Universal Biosensing Systems Based on Monoclonal Antibody." **Smita Joel**, Anjan K. Bhattacharyya, Boyd Haley, Leonidas Bachas, and Sylvia Daunert.

3. Poster presentation at the Kentucky Academy of Science, November 2009, Northern Kentucky University, KY, United States. "Fiber Optic Biosensor for *In situ* Monitoring of Hydroxylated PCB's." Neha Ray, **Smita Joel**, Kendrick Turner, and Sylvia Daunert.

4. Poster presentation at the University of Kentucky for NSF Research Experiences for Undergraduates 2009, Lexington, KY, United States. "Enhancing Natural Fluorescence: Incorporation of 7-azatryptophan into Glucose Binding Protein." Trajen Head, **Smita Joel**, and Sylvia Daunert.

5. Poster presentation at the 14th Annual EPSCoR Conference, October 3, 2008, Louisville, KY, United States. **"**Biosensors Based on Site-Specific Labeling of Antibodies." **Smita Joel**, Boyd E. Haley, Leonidas G. Bachas, and Sylvia Daunert.

6. Poster presentation at the University of Kentucky for NSF Research Experiences for Undergraduates August 2008, Lexington, KY, United States. "Creating a

Glucose Biosensor: Global Incorporation of 7-azatryptophan in Glucose Binding Protein." Jacqueline Ward, **Smita Joel**, and Sylvia Daunert.

7. Poster presentation at the Clinical and Translational Science Conference, June 3, 2008, Lexington, KY, United States. "Reagentless fiber optic biosensors for the continuous monitoring of glucose." **Smita Joel**, Lisa Moschou, and Sylvia Daunert.

8. Poster presentation at the Naff Symposium, April 4, 2008, Lexington, KY, United States. "Reagentless fiber optic biosensors for the continuous monitoring of glucose." **Smita Joel**, Lisa Moschou, and Sylvia Daunert.

9. Poster presentation at the University of Kentucky for NSF Research Experiences for Undergraduates August 2007, Lexington, KY, United States. "Incorporation of Unnatural Amino Acids in Glucose Binding Protein: Developing a Thermally Stable Glucose Biosensor for *in vivo* Applications." Eric Hunt, **Smita Joel**, Kendrick Turner, and Sylvia Daunert.

10. Poster presentation at the 39th Central Regional Meeting of the American Chemical Society, May 20-23, 2007, Covington, KY, United States. "Reagentless fiber optic biosensors for the continuous monitoring of glucose." **Smita Joel**, Lisa Moschou, and Sylvia Daunert.

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