Chapter 1. Introduction

1.1 Motivation

Biosensors, devices used to identify the presence or concentration of a target biomolecule, have become a pervasive tool in medical diagnostics.[1-3] Because these sensors see frequent use in early-stage detection of cancer and diseases, applications for which the concentration or change in normal body concentration of the intended analyte is low,[4-5] it is critical that biosensors have a high sensitivity for the target molecule. Additionally, these devices are commonly used at the point-of-care, where rapid, simple, and inexpensive testing is a necessity.[4-6] Despite the need for biosensors that can provide rapid and accurate readings directly on samples acquired from patients, most sensors display limited success at this feat. One of the most common issues is reduction in sensitivity due to nonspecific binding effects.[7-8] Because biosensing fluids usually contain off-target molecules millions of times more concentrated than the intended analyte, the large number off-target molecules bind to sensor surfaces, blocking analyte receptor sites and reducing device sensitivity.[9-10] Some techniques have been developed to overcome these nonspecific binding issues, but these methods rely on time-consuming or expensive sample pre-treatment.[11-12]

Therefore, this thesis aims to develop a freestanding biosensor platform that can achieve high sensitivity and selectivity for a target analyte in biological fluids and other complex mixtures. The remainder of this chapter will provide a literature review of concepts and methods utilized in the design of this biosensing platform. Beginning with a comprehensive summary of the active components in biosensors, topics will gradually become increasingly focused on the selected biosensor design. The chapter will conclude with a presentation of the material selected for biosensor development and an overview of the research projects undertaken to develop and optimize this biosensor.

1.2 Proteins

The most fundamental components of biosensors that give these devices sensing capabilities, proteins are a diverse set of biological macromolecules consisting of one or more amino acid chains folded into complex 3D architectures. The process of protein folding, though occurring over times as short as microseconds,[13] is incredibly complex and allows a sequence of amino acids to fold into a unique structure. During folding, nearby amino acids first sample different conformations by forming local metastable structures through hydrogen bonding and other specific intramolecular interactions.[13] As the folding process progresses, many of these higher-energy metastable structures unfold while more stable regions grow and combine with adjacent structures until the peptide chain ultimately folds into its native state.[13-14] Though the number of intramolecular interactions that govern protein folding is rather limited, hundreds of different classes of local chain topologies exist in native protein structures,[15] and 5 million unique proteins are estimated to exist on Earth.[16] This great diversity of structures has given rise to proteins with a wide range of functions, enabling use of these biomolecules in a myriad of processes and products, including food processing and packaging,[17-20] pulp and paper bleaching,[21] detergents,[17, 22] and therapeutics.[23-24]

In many cases, the structure of a protein allows it to bind with high specificity to a substrate. Proteins have been found to bind to a variety of molecules, including small molecules,[25-27] DNA/RNA,[28-30] and other proteins.[31-32] Traditional models for protein-substrate binding mechanisms have either assumed that the protein structure is completely rigid and the substrate fits neatly into some binding site on the protein surface (i.e., the lock-and-key model)[33] or that the binding site is somewhat pliable and the substrate is able to slightly distort the biding site to fit the structure of the substrate (i.e., the induced fit model).[34] While many proteins do bind substrates

according to these models that assume a completely or almost completely rigid protein structure, studies within the past two decades have revealed that numerous proteins contain disordered regions of at least 10 amino acids in length, yielding more dynamic protein structures.[35] These disordered regions can allow a protein to adopt one or several conformations to achieve high-specificity binding to substrates.[36-37] For example, ubiquitin contains a large, highly dynamic region within its sequence, and this protein is known to form over 50 unique structures, each of which can bind to a distinct protein.[37-39] An extreme case of dynamism, intrinsically disordered proteins (IDPs) are proteins with little or no well-defined, persistent structure.[40-41] Despite having no true structure, IDPs can partially fold and form a strong bond in the presence of a substrate or other binding partner,[40-41] though no single hypothesis has emerged regarding the mechanism of this folding.[42-44]

Enzymes are another class of proteins that not only bind to a substrate, but facilitate a change in the structure of the substrate by catalyzing a chemical reaction. This catalysis is primarily achieved through interactions between specific residues in the active site, the region in the enzyme in which the chemical reaction occurs, and the substrate.[45] For instance, one very common set of residues that combine to provide catalytic activity is the catalytic triad, which is composed of a nucleophilic residue (e.g., serine), a basic residue (e.g., aspartate), and an acidic residue (e.g., histidine). Mechanisms involving this catalytic triad proceed by the base deprotonating the nucleophile, the acid stabilizing the resulting salt of the base, and the activated nucleophile attacking an electrophilic site on the substrate.[46] Though a wide variety of other catalytic series of residues exist, including unconventional modifications to the catalytic triad,[47] the interactions between the residues usually provide either charge, and acid/base, a nucleophile, or tautomerization.[45] In some enzymes, cofactors such as metal ions and small organic molecules are

also required for reaction to occur, typically to either stabilize the structure of the enzyme or the transition state in the chemical reaction.[48-50] While residues and cofactors within the active site directly catalyze the chemical reaction, recent studies have found that even residues far from this site affect catalysis rates.[51-53] Due to the non-static nature of the structure of enzymes and other proteins, it is believed that dynamic fluctuations, such as those caused by solvent molecules, affect the motion of individual residues, and this vibration is propagated to the active site, resulting in conformational changes.[54-56]

With the rapidly growing understanding of protein structure and function, many proteins can now be engineered for specific applications. One method to achieve unique protein functionality is through installing non-canonical amino acids by providing cells with modified tRNA molecules during recombinant protein expression.[57] Using this technique, proteins have been designed to understand protein structure and function,[58] provide new spectroscopic properties,[59] install site-specific groups for post-functionalization,[60] and modulate the clearance time of bacteriophages in plasma.[61] Protein scaffolds, proteins with well-defined binding faces that can easily be modified to display specificity for an arbitrary substrate, have also been identified for engineering novel proteins. From just a small number of scaffolds, proteins have been developed for therapeutic applications,[62-64] with enhanced thermal stability,[65] and to direct self-assembly of other molecules.[66] When an enzyme is desired for a specific reaction but it is not clear what mutations need to be made to an existing protein to achieve the desired functionality, directed evolution can be used. In this method, the gene sequence of an enzyme is subjected to successive rounds of mutagenesis. The activity of the mutants for the desired reaction is then measured and, in a manner similar to natural selection, used to direct future mutations towards the desired enzyme function.[67-68] This technique, though requiring no knowledge of what the final structure of the

engineered protein should be, has allowed development of numerous enzymes with industrially relevant applications.[69-70] Clearly, the breadth of protein function is expansive, and this diversity is continuously increasing.

1.3 Protein Biosensors

One of the largest applications for binding proteins is in protein biosensors. Biosensors are devices used to detect the presence or concentration of one or more biological analytes. In general, these sensors contain three primary components: an element that selectively recognizes the target analyte(s), typically through a binding event, a part that converts this recognition event into a measureable signal, and a method for either quantifying the measurable signal or converting this signal into a useful and more easily understood form.[71] As an example, one of the earliest developed biosensors was a blood glucose sensor containing a thin film of glucose oxidase coated on an oxygen electrode.[72] A patient's blood was passed through the machine, where glucose oxidase catalyzed the consumption of glucose and oxygen. The corresponding drop in dissolved oxygen was detected at the electrode, and the change in potential was compared against a standard curve to provide a blood glucose concentration. While the technology used in this blood glucose sensor was simple, the design principles formed the foundation for the now burgeoning field of biosensors.[1-3]

Today, there are a wide variety of biosensor formats that use surface-immobilized proteins as the molecular recognition elements. Surface plasmon resonance (SPR) biosensors are widely used designs that consist of receptor proteins for a target analyte immobilized on a gold surface.[73-75] When analyte molecules bind to these capture proteins, the refractive index at the surface of the sensor changes, affecting the propagation rate of surface plasma waves that are generated when the surface is exposed to plane-polarized light. Changes in this propagation rate can be indirectly

measured to infer protein binding to the surface, but changes in signal can also be caused by binding of off-target proteins to the sensor surface or changes in refractive index due to temperature and concentration fluctuations.[76] Quartz crystal microbalance (QCM) biosensors have been designed that use piezoelectric effects to detect changes in mass corresponding to analyte binding.[77-80] Microcantilever biosensors operate on a similar principle, in which analyte binding to capture proteins on the surface of microcantilevers shifts the resonance frequency of the cantilever, allowing quantification of protein binding.[81-85] Yet another class of sensors, lateral flow assay (LFA) biosensors[86-87] and microfluidic biosensors[88-89] are typically paper-based devices that use capillary action to wick samples across regions where the analyte is sequentially labeled (e.g., with a dye) and bound to a capture protein. These biosensors offer a combination of low cost and high sensitivity that has prompted their use in point-of-care applications.[90-92]

Despite the diversity in their design, biosensors incorporating surface-immobilized proteins all have two major issues. First, capture proteins must be immobilized to the sensor surface with proper orientation such that the binding sites in the proteins are fully accessible.[93-95] Typical immobilization techniques produce a random orientation of proteins on the surface. While numerous procedures have been reported to achieve well-oriented protein monolayers,[96-101] these methods all require either pretreatment of the protein/surface or that the protein have a specific structure. Second, nonspecific binding from molecules present in the detection fluid along with the intended analyte increases the background signal for biosensors, reducing sensitivity by up to several orders of magnitude.[9-10] Due to this significant issue, a variety of both passive[102] and active[103] methods for reducing nonspecific binding have been developed. However, these methods either reduce sensor longevity or add complexity, making it challenging to use biosensors for simple and rapid analysis. Thus, the development of general methods for properly orienting capture

proteins and reducing nonspecific binding are of high priority for optimizing biosensor performance.

1.4 Block Copolymer Self-Assembly

To orient capture proteins, inspiration can be drawn from block copolymer self-assembly. Block copolymers provide a general framework for creating 3D arrays of densely packed and well-oriented materials. While a variety of block copolymer architectures have been developed, including nonlinear,[104] multiblock,[105-106] and tapered block copolymers,[107-108] the simplest and most well-studied design is the diblock copolymer. These copolymers, composed of two homopolymers linked together end-to-end, have been demonstrated to self-assemble into a variety of nanostructures including lamellae, hexagonally-packed cylinders, and gyroids.[109-112] Phase separation within diblock systems is driven by three parameters: χ, the Flory-Huggins interaction parameter, which is an enthalpic term describing the degree of chemical dissimilarity between the two blocks, N, the overall degree of polymerization, which encompasses the entropic penalty of chain stretching, and f, the volume fraction of one of the blocks.[109-111] The onset of phase separation for symmetric diblocks is predicted to occur at $\chi N = 10.5$, at which point the entropic penalty associated with stretching chains in the ordered state is balanced by the decreased enthalpy as a result of decreased interfacial area.[111] Because the two blocks are connected by a covalent bond, macrophase separation does not occur, and the blocks instead separate at lengths scales on the order of 10-100 nm.[110]

In dilute aqueous solution, amphiphilic diblock copolymers primarily form micelles, structures in which hydrophobic blocks aggregate in solvent-poor phases surrounded by hydrophilic blocks extending into a solvent-rich phase. While individual block copolymer molecules are distributed throughout the solution and act as surfactants at low concentrations,

micelles spontaneously form above a critical micelle concentration (CMC).[113] Above the CMC, micelle stability is primarily controlled by the hydrophobic block, where longer and more hydrophobic chains significantly enhance stability.[114-115] Varying the relative length of the hydrophilic block yields a variety of morphologies, including bilayers, cylinders, wormlike micelles, and spheres;[116-117] the self-assembly of spherical micelles in particular has garnered significant attention in the literature.[113, 118-120]

In concentrated solution, diblock copolymers self-assemble into many of the same phases observed in the bulk state. Unlike in the bulk state, however, the solvent and therefore its selectivity for each block can be adjusted in concentrated solution, providing another variable by which phase behavior can be controlled.[121-123] Since a perfectly neutral solvent distributes equally between domains, it is assumed that the only effect of the solvent on segregation strength is to dilute monomer-monomer interactions in proportion to the polymer volume fraction, which is referred to as the dilution approximation.[124] Selective solvents not only dilute monomer-monomer interactions but also selectively partition into one phase, effectively changing the volume fraction of each block. When these solvent effects are mapped onto a bulk diblock copolymer phase diagram of χN vs. f, good qualitative agreement is shown with the bulk data, except that the region of disordered phases is expanded in solution.[121, 125] It has also been demonstrated in both theory[126] and experiments[127] that solvents that are good and nonselective for both blocks preferentially segregate to the interface, shielding the repulsive interactions between phases and causing a reduction in overall domain spacing.[128] This enhanced solvent distribution at the interface between phases is not observed in nonselective solvents,[127] where domain spacing trends with polymer volume fraction can vary greatly as a function of both temperature and the assembled nanophase.[128]

In block copolymer thin films, films thinner than approximately one micron, the small length scales cause morphology to be largely determined by confinement and interfacial effects. For example, block copolymer thin films that assemble into lamellae can be oriented either perpendicular or parallel to the underlying substrate. Because lamellae have a natural repeat spacing, parallel lamellae pay an energetic penalty due to chain stretching or compression if film thickness is not commensurate with this spacing, and perpendicular lamellae are favored.[129-131] However, if the surface energetics at the top and bottom interfaces of the film (e.g., the underlying substrate and the atmosphere) are different such that one block preferentially segregates to each interface, a parallel lamellar orientation is more stable.[132] These surface effects can be used to induce ordering that propagates hundreds of nanometers into thin films,[133-134] as has been realized in several robust methods for inducing order such as epitaxy[135] and solvent vapor annealing.[136] The interplay between confinement and surface energetics can result in thin films displaying islands/holes[112, 137] and morphologies that vary drastically with even small changes in film thickness.[138] As such, block copolymers offer a wealth of methods to achieve self-assembled materials.

1.5 Protein-Polymer Conjugate Self-Assembly

Protein-polymer conjugates, block copolymers in which one block is a protein, exhibit the self-assembly properties of traditional block copolymers while also introducing biological functionality. In dilute solution, amphiphilic protein-polymer conjugates have been shown to self-assemble into micellar morphologies, as is observed in coil-coil diblock copolymers.[139-141] These micelles have primarily been used for drug and gene delivery applications,[142-143] where the proteins blocks can promote intracellular delivery,[144] enable stimuli-driven changes in micelle size and density,[145] and both bind and release payloads.[146] Furthermore, the protein block in protein-

polymer conjugates can enhance self-assembly if the protein itself forms or promotes well-defined structures such that hydrophobic collapse of one block is no longer required for self-assembly in aqueous solution. For instance, when peptides that form beta sheets are conjugated to polymers, these beta sheets promote fiber formation.[147-149] Similarly, coiled-coils, supercoiled bundles of alpha helices, have been conjugated to polymers to promote formation of aggregates with precise aggregation number[150-151] as well as rodlike and spherical micelles.[152] When several of these coils are incorporated in the protein block of protein-polymer block copolymers, hydrogels can spontaneously form from association of coiled-coil domains.[153-156]

In bulk and concentrated solution, the self-assembly of proteins conjugated to a fully-folded protein block shares some similarities with that of coil-coil diblock copolymers. Like traditional diblock copolymers, protein-polymer conjugates can access a wide range of morphologies, including lamellae, hexagonally packed cylinders, and gyroids.[157-159] These periodic structures with regions of densely packed protein enhance protein activity, as realized in biocatalysts and biosensors with significantly improved performance compared to devices with surface-immobilized proteins.[160-161] Unlike traditional coil-coil block copolymers, however, protein-polymer conjugates display lamellar ordering over a wide compositional range, asymmetric phase diagrams, and re-entrant order-disorder transition (ODT) behavior.[162-163] Though the cause of these behaviors is not fully understood, recent work has demonstrated that the identities and ordering qualities of observed protein-polymer nanophases are highly dependent upon coarse-grained properties of the protein block (i.e., size and structure) and attributed entanglement effects from the polymer block.[164] Despite an incomplete understanding of the self-assembly of protein-polymer conjugates in concentrated solution, the enhanced activity and

sensitivity exhibited by these materials provides significant motivation to study their use in biotechnological applications.

1.6 Transport in Block Copolymer and Homopolymer Solutions

While the self-assembly properties of protein-polymer conjugates can be utilized to orient capture proteins in biosensors, the transport properties of these block copolymers can also allow minimization of nonspecific binding. To date, the diffusion in ordered block copolymer solutions has primarily been studied for two classes of diffusants: block copolymers (self-diffusion) and ions. For diblock copolymer self-diffusion in lamellae, diffusion can occur either along the interfaces between domains (parallel diffusion), in which each block of the copolymer remains within its own phase, or through the lamellar layers (perpendicular diffusion), where blocks must pass through domains of the opposite phase. Clearly, perpendicular diffusion is expected to pose a much greater resistance to diffusion of the diblock copolymer, as it requires enthalpically unfavorable movement of the blocks of the copolymer into chemically dissimilar domains. Indeed, early diffusion experiments in disordered and lamellar block copolymers without macroscale orientation showed that the diffusion of Rouse chains was essentially unaffected by ordering, whereas reptating chains exhibited significantly reduced diffusion in the ordered state.[165-167] Direct measurements of these two methods of diffusion were later used to confirm that the diffusion coefficient for parallel diffusion is over an order of magnitude greater than that for perpendicular diffusion in lamellae.[168] Similar asymmetries have also been measured in other block copolymer morphologies.[169]

For ion diffusion in block copolymers, diffusion is considered to occur within a single domain of the system. As such, diffusion through randomly oriented grains of morphologies in which diffusion primarily occurs along one or two of the principle directions, such as cylinders

and lamellae, is expected to be reduced by a factor of 1/3 or 2/3, respectively, compared to bulk diffusion. These morphology-dependent factors for diffusion reduction have been supported by both theory[170] and experiments.[171-173] Morphologies with more complex symmetries such as gyroids instead reduce diffusion by a factor intermediate to 1/3 and 2/3, for which the exact reduction is dependent on the relative volume fraction of each phase.[174]

While self-diffusion and ion transport in block copolymer solutions have been well-studied, the diffusion of nanoparticles and proteins in these solutions is almost completely unexplored. At the time of writing, only one study has reported on this topic, in which it was demonstrated that protein diffusivity in micelle crystals was reduced by up to four orders of magnitude compared to diffusivity measurements in aqueous solution.[175] Instead of focusing on diffusion in block copolymers, most research has considered diffusion of nanoparticles and proteins in homopolymer solutions and gels. For nanoparticles, it has been found that the diffusivity of the particle is dependent upon the size of the particle relative to the radius of gyration (R_g) of the polymer.[176] While diffusion follows the Stokes-Einstein relation if the nanoparticle is larger than the polymer R_g, the particle diffuses much faster than is predicted by this equation if the particle is smaller than the polymer R_g.[177-179] Theories have been developed to rationalize this phenomenon, which have assumed diffusion mechanisms including nanoparticle hopping between entanglements[180] and fast diffusion within a confined depletion region at small length scales.[181-182]

Unlike nanoparticles, which are assumed to not interact with polymer chains, proteins can and do interact with polymers, leading to more complex transport phenomena. Polymers such as dextran that interact with proteins increase the effective hydrodynamic diameter of the protein, causing negative deviations in translational diffusion from Stokes-Einstein predictions.[183-186] Rotational diffusion also is lower than expected, though different studies find that the decrease in

rotational diffusion can either be greater than or less than that of translational diffusion.[184-186] Even polymers that do not interact with proteins, however, can still affect diffusion by indirectly controlling protein-protein association. When these non-interacting polymers are in the dilute regime, the polymers induce preferential hydration of the proteins, reducing the likelihood of aggregation.[185, 187] In the semidilute regime, association rates are enhanced, while in the concentrated regime, protein-protein association is again diminished, which has been attributed to depletion affects.[187] Thus, diffusion of proteins in polymer solutions is incredibly complex, depending upon the identity and concentration of both the protein and polymer.

1.7 Thesis Overview

Due to their potential to orient capture proteins and control molecular transport, protein-polymer conjugates were selected as an ideal material for biosensor fabrication. Thus, the goal of this thesis is to develop strategies to design protein-polymer conjugate biosensors with enhanced sensitivity and selectivity in complex sensing mixtures. To accomplish this objective, techniques to improve conjugate self-assembly will be presented, and factors governing transport within these the fabricated biosensors will be studied. In this document, chapter 2 provides detailed methodologies for the synthesis, data collection, and data processing used in the thesis. Chapters 3 and 4 discuss methodologies for enhancing ordering quality of protein-polymer conjugates through genetic engineering of the protein block. In chapter 3, oligomerization of the protein block is explored as a method to promote better assembly of otherwise weakly ordered conjugates with low molecular weight protein blocks. Chapter 4 presents a more general ordering technique of fusing associative heterodimeric coiled-coil sequences to the protein block. In chapter 5, the biosensing capabilities of bioconjugate thin films are measured and compared to those of traditional surface-immobilized protein biosensors. Performance, including ability to prevent nonspecific binding during sensing

in biological fluids, is also measured as a function of the molecular weight of the polymer block in the protein-polymer conjugate. Finally, chapter 6 explores factors affecting size-based transport of proteins in conjugate thin films. Diffusion coefficients for a panel of proteins is measured in protein and polymer solutions individually, and the results are compared to diffusion measurements in the thin films, providing insight into the relative diffusional resistance applied by each block.

1.8 References

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