DEVELOPMENT OF A FLUORESCENCE-BASED PROTEASE BIOSENSOR USING NANOSCALE PLATFORMS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DEVELOPMENT OF A FLUORESCENCE-BASED PROTEASE BIOSENSOR USING NANOSCALE PLATFORMS

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ABSTRACT

The development of a protease biosensor utilizing nanomaterials is presented in this dissertation. Peptide substrates and nanomaterial platforms were investigated to increase the sensitivity and response time for the detection of protease analytes. Nano-sized platforms, including nanoparticles and nanofibers, offer the advantage of a higher surface area-to-volume ratio contributing to increased immobilization points and a capability of an enhance signal output. Nanoparticles (Silica Nanoparticles, Quantum Dots, and Gold Nanoparticles) in solution and solid surface (Polymer) nanofibers were tested with immobilized peptide substrates that contained fluorophores to acquire fluorescence for signal transduction. A full characterization was accomplished through a variety of optical characterization techniques and an optimized protocol has been developed for each of the sensing systems. The results from these studies are reported for each of the sensing platforms with response to protease analytes.

CHAPTER 1

LITERATURE REVIEW

1.1 Biosensors

Sensing systems utilizing a variety of platforms have been developed for the detection of a myriad of analytes for many years. The development, operation, and application of sensors have been investigated to detect targeted species with speed and accuracy. These principles of sensing platforms and sensing mechanism with relation to optical biosensing are being investigated to improve overall sensor performance.

Biosensors are devices that detect analytes using a biorecognition receptor which can operate by turning this interaction into a usable signal (signal transduction) (D'Orazio 2003). Optical, electrochemical, or piezoelectric sensing are a few of the main classes of biosensor transducer systems (Prusaksochaczewski et al. 1990; Takhistov 2004). Some of the most common analytes that have been detected are biomarkers in the blood, glucose, food contaminants, explosives and bio-threat agents (Bonanno and DeLouise 2007; Ko and Grant 2006; Medintz et al. 2005a). Array biosensors are also being developed to detect multiple analytes simultaneously (Sapsford et al. 2006).

Health care is one of the primary applications for biosensors (Baldini 2005). The ability to selectively and reliably measure blood analytes, gases, ions, and metabolites is very essential to show a patient's metabolic state and to sense potential threats (Vo-Dinh and Cullum 2000). Currently blood or urine samples need to be taken to a lab for analysis, which could take hours and sometimes days. These standard methods are

typically very sensitive, but they are time-consuming and expensive. Ideally biosensors could be implanted in the body and continuously monitor metabolite levels in real time. These sensors could also be coupled with a drug delivery system that could provide accurate therapies as needed (Tan et al. 2004). The problem with many of these devices is that biofouling is likely to occur (Ratner 1996). Most current research is involved in making these implantable devices biocompatible. The idea of miniaturizing biosensors down to the nanoscale has been an important advancement in this technology (Shipway et al. 2000). The development of a highly sensitive biosensor that is biocompatible would have an extraordinary impact on the future of patient care.

The need for accurate, rapid, and inexpensive detection methods has lead researchers into exploring other options, including new sensing mechanisms and sensing platforms. New enabling tools are being developed to improve sensor detection. Point-of-care systems are designed to operate alongside a patient's bedside supplying automated diagnostic and laboratory testing and they are utilized to diagnose an array of different ailments (Soper et al. 2006). They can also provide necessary information to assist in prognosis and treatment. For example, Soper has developed a point-of-care biosensor system for cancer detection which requires a large panel of markers.

Advances in nanotechnology have lead to new generations of sensing probes with extremely small sizes that make them ideal for monitoring physiological and biological processes (Vo-Dinh 2008). Integrated sensing systems are also being developed that can very quickly detect analytes of interest remotely and with little expense (Sapsford et al. 2006; Thrush et al. 2003). These integrated devices are sometimes referred to as "Lab-

on-Chip" devices and like biosensors must be able to detect and quantify the presence of an analyte (Soper et al. 2006; Toner and Irimia 2005).

Nanotechnology has significantly boosted the field of sensors. Making sensors smaller and integrating platforms has the potential to reduce costs and power needs. Much work is being done in the area of nanosensors for the detection of diseases and cancers to name a few (Soper et al. 2006). This technology can revolutionize the way patients are evaluated and treated with applications both in the medical industry and homeland security (Vo-Dinh et al. 2006).

1.2 Sensing Mechanism

The mechanics by which the analyte and the sensing probe function is termed the "sensing mechanism". This process occurs due to the binding and the interaction of the analyte on a biorecognition element. This creates the signal that must be transduced into a useable signal. Signaling mechanisms including antibody-antigen binding in immunoassays, formation of DNA chains, and enzymatic cleavage for proteolytic assays as well as others, have all been previously studied (Fan et al. 2005).

Affinity of potential analytes for a given target must be studied for each instance, as the molecular recognition of different analytes will function and react differently in different conditions. A few basic detection methods have been generalized by (Iqbal et al. 2000) stating nucleic acid-based detection is more specific and sensitive than immunological-based detection, but that the second is faster and more robust (Iqbal et al. 2000). Sensing techniques have also changed with the emergence of synthetic substrates,

which were compared to anti-body based receptors in a paper by (Vikholm-Lundin et al. 2008), who wants to replace anti-bodies with robust and inexpensive synthetic receptors.

The most common sensing mechanisms are nucleic acid based, immunoassays, and proteolytic cleavage. An example of nucleic acid-based detection relies on coassociation of two aptamers recognizing two distinct epitopes of a protein (Heyduk and Heyduk 2005). These aptamers can be fluorescence based for signaling. The immunoassay technique is one of the most popular and involves an antigen binding to an anti-body to elicit a measurable response. Examples of this immunosensor technique include the well-known enzyme-linked immunosorbent assay (ELSIA) used to detect antigens and antibodies (Han et al. 2007). Lastly proteolytic cleavage is a technique that involves a peptide substrate that is designed to cleave in the presence of a target analyte with high specificity (Shone et al. 1993).

1.2.1 Biological Recognition Elements

Bioreceptors bind target analytes and allow for interaction of analyte with the sensing probe (Soper et al. 2006). Antibodies, enzymes, nucleic acids substrate-based probes are some of the most common recognition elements used in biosensor development (Endo et al. 2005; Ligler et al. 2002; Miscoria et al. 2005; Watanabe and Ishihara 2008). A requirement of biorecognition elements is that they are covalently immobilized to the surface of the transducer and that they allow the activity of analyte to take place. Packing density is also of great importance to allow for a smooth interface for sensing and to minimize any steric hindrance (Wink et al. 1997).

Antibody systems are highly selective to a target antigen and antibodies can be raised for a variety of species (Vikholm-Lundin et al. 2008). Immunoassays function by immobilizing antibodies on the surface and are dependent on the orientation in which they bind. Enzyme substrate-based receptors have high specificity and require immobilization of the biocatalyst and transformation results in either an optical or electrical signal (Choi 2004). Nucleic acids have been used but have difficulties in achieving homogenous surface coverage, reliable orientation of the molecule, and access to base pairs to deem it as an optimal sensing surface (Breimer et al. 2003).

A new trend is to replace antibodies with robust and inexpensive synthetic receptors. Peptides are short strands of amino acids and can be synthesized with any number of sequences to function as synthetic receptors that can interact with a potential analyte with a high degree of selectivity. For protease analytes, which bind and cleave a target receptor, a peptide substrate can be designed that mimics this receptor and the cleavage process can be leveraged as sensing mechanism which can be transduced into a usable signal. These fluorogenic peptide substrates have been demonstrated with great success to detect traces of proteolytic activity (Grahn et al. 1998).

Phage display libraries have recently been employed to determine synthetic receptors for many different analytes (Goldman et al. 2000). These libraries operate by indentifying the substrate that selectivity binds to the target analyte (Soper et al. 2006). For instance, Goldman found a selection of phage displayed peptides for the detection of 2,4,6-tirnitrotooluene (TNT) derivative for a continuous flow sensor (Goldman et al. 2002). An amino acid sequence that binds anthrax has also been found by screening a

phage display peptide library (Williams et al. 2003). Additionally these peptides have also been previously utilized with cantilever array platforms for vapor detection with good selectivity (Lim et al. 2006; Shu et al. 2008).

1.2.2 Optical Detection

Optical detection can use fluorescent wavelengths, intensity values, and lifetime to measure analyte binding and activity. Interactions between the biorecognition molecule and a target analyte illicit an optical signal which can be collected and transduced (Cheung et al. 2008). The intensity of this signal is usually in arbitrary units but can be normalized and compared to assess a sensing system. Fluorescence assays directly measures the analyte function and requires no further tracer reagent in order to acquire the signal. In many cases the fluorescent signal detected is directly proportional to the analyte concentration.

Biosensing systems based on optical detection have been utilized for their advantage of being resistant to electromagnetic interference, capability of remote sensing, and multiplexed detection with a single device (Fan et al. 2008). Optical biosensors can consist of label-free based detection as well as fluorescence (labeled) based detection.

Fluorescence based optical detection easily incorporates small fluorescent labels that enhance sensitivity and serve as a powerful analytical tool to detect and quantify various analytes using specific biorecognition receptors. Fluorescence can be leveraged in many ways and can be utilized for signal collection and analysis. For example, fluorescence resonance energy transfer (FRET) is an optical phenomenon that occurs between two fluorescent molecules that are in close proximity and have an emission

overlap of the others excitation wavelength (Medintz et al. 2003b; Wu and Brand 1994). FRET is a non-radiative transfer of energy from an excited state donor molecule to an acceptor molecule and offers the advantage of real-time ratiometric quantification of analyte interaction of the peptide substrate (Dacres et al. 2008; Grant et al. 2004b; Grant et al. 2005b). FRET was described by Forster in 1959 and is now used widely as a research tool (Lakowicz 1999). The schematic for FRET between two florescence molecules in close proximity and then separated with their characteristic wavelength emissions are shown in Figure 1.1.

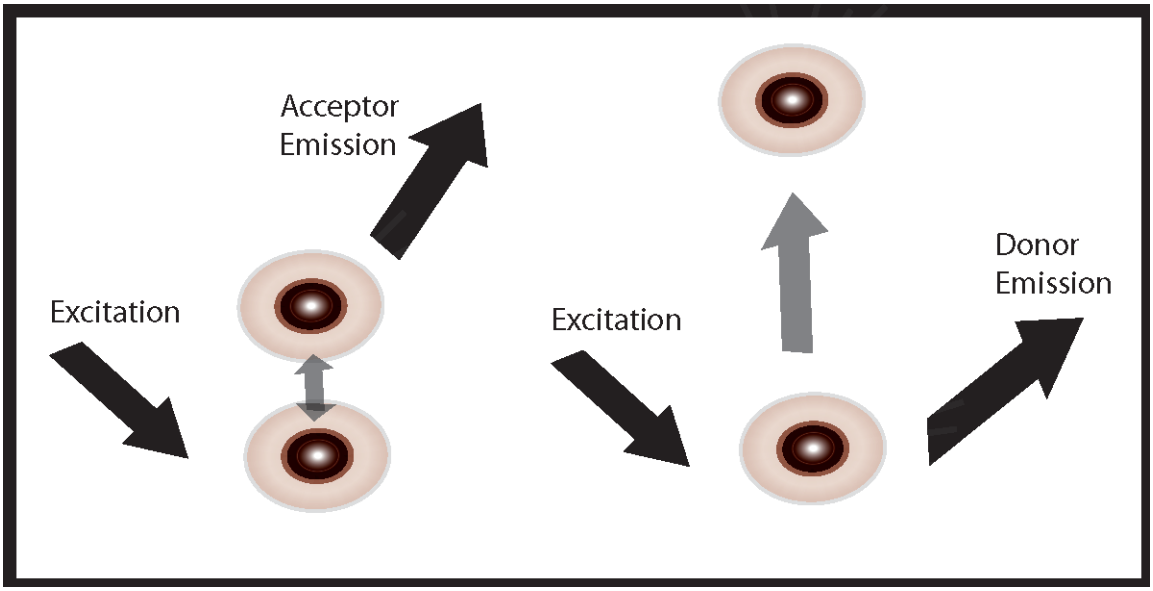

Figure 1.1 – Schematic of FRET between fluorescence molecules with respect to their proximity and their characteristic wavelength emission

Fluorescence quenching is another method by which a fluorescent molecule can transfer its energy to another molecule which absorbs the energy and does not radiate emission as fluorescent light.

1.3 Sensing Platforms

Using plain glass slides or well-plates to run laboratory assays is becoming obsolete in the biosensing industry. Recent advances in the design and fabrication of sensor platforms using micro and nanotechnology has enabled sensing systems with higher sensitivity (Liang et al. 2005; Takhistov 2004). Microfabricated devices are among the platforms having the largest impact with applications in clinical diagnostics to security applications (Baldini 2005). These devices usually made of polymers are small in size, easily produced, portable, and need a minimal sample volume (Sapsford et al. 2008a). All these properties significantly can reduce the cost of small scale devices with optimized assays performance. Additionally sensing platforms that are reusable and that are capable of detecting multiple samples simultaneously are highly desired. Multi-analyte analysis with patterned surfaces can test for multiple different analytes and at the same time decrease the assay time (Golden et al. 2005; Rowe-Taitt et al. 2000). Rowe-Taitt has extended their prior technology to detect six biohazardous agents on a planar waveguide array biosensor.

Planar waveguides have evolved greatly due to the coupling of these devices with polydimethylsiloxane (PDMS) flow cells which allow for control of microfluidic testing platforms (Chang-Yen et al. 2005; Rowe-Taitt et al. 2000). Microfluidics is the manipulation of liquids or gases through a system on the micro-scale (McDonald et al. 2000). Layer-by-layer assembly of many sensing platforms has optimized designs to create sensor chips with both sensitivity and multi-analyte detection. (Shenhar and Rotello 2003) described a few unique structural arrangements of nanoparticles for

devices with tunable specificity and environmental response. For example, controlling nanoparticle monolayers can create scaffolds for device fabrication of sensors. And a single step, bottom-up technique based on conducting polymer nanofibers has been developed that are faster and have 4 times larger current variations than bulk sensors (Li et al. 2008). Lastly liquid core waveguide-based fluoresce detection systems have been developed for measurement of trace amounts of analytes in small sample volumes (<1µL) (Song et al. 2008).

Materials on the nanoscale whether nanoparticles in solution or nanoporous coatings offer key advantageous for increasing the sensitivity of a sensing system (Rossi et al. 2007). Nanomaterials increase the surface-to-volume ratio because the smaller the diameter/dimensions of any material the total volume decreases faster than the surface area. Sensing platforms built with nanomaterials will contain a higher surface area-to-volume and thus allow more area for biorecognition molecules to bind and an expanded surface for analytes to interact. This will lead to lower limits of detection and faster responses.

1.3.1 Silica Nanoparticles

Silica nanoparticles are glass beads that can be synthesized with various diameters, from 1-100nm (Rao et al. 2005). They are monodispersed (water soluble) and the surface of silica nanoparticles can be treated like glass and can be easily modified for attachment to biomolecules using simple silanization protocols (Qhobosheane et al. 2004). Silanization is a method to covalently immobilize functional biomolecules to the surface of silica substrates using thiol-terminal silanes and hetero bifunctional cross-

linkers (Bhatia et al. 1989). Nanoparticles provide a large specific surface area and work well as a sensing platform capable of binding biorecognition molecules (Watanabe and Ishihara 2008). Some applications of silica nanoparticles include doping them with large numbers of dye molecules and using them as a sensing platform. The silica matrix protects these fluorophores from environmental oxygen and so are much more photostable (Liang et al. 2005; Ow et al. 2005). These dye-doped silica nanoparticles have been used as multicolor FRET barcoding tags for multiplexed signaling (Wang and Tan 2006). (Tan et al. 2004) summarized some of technologies based on silica nanoparticles, including bioconjugation with enzymes, antibodies, and DNA molecules, and their applications for biosensing and bioimaging.

1.3.2 Quantum Dots

Quantum dots, or semiconductor nanoparticles, have unique properties that make them especially well-suited as fluorescent labels in fluorescence assays (Sapsford et al. 2008a). They exhibit a high quantum yield and resist photo-bleaching. Their narrow size-tunable photoluminescent emissions and a broad absorption spectrum make them ideal FRET donors. The semiconductor core radius is smaller than the bulk excitation Bohr radius which leads to discrete energy levels allowing size tunable photoelectron emission (Liang et al. 2005). They can also serve as a nanoscale platform for attaching biorecognition receptors with acceptor labels in fluorescence assays (Alivisatos et al. 2005; Vu et al. 2005). Some concerns for quantum dots are their inherent problem with blinking, unknown toxicity, and unstable nature.

Quantum dots have been used in self-assembled nanoscale biosensors acting as FRET donors and as fluorescent labels for cellular imaging (Gao et al. 2005; Jain and Stroh 2004; Lagerholm et al. 2004; Medintz et al. 2003a; Parak et al. 2005; Stringer et al. 2008; Zhang et al. 2005). The report by Medintz describes the preparation and testing of a quantum dot-protein/receptor nanosensors for recognition-based sensing. This study was later utilized to detect proteolytic activity using peptide conjugates measuring enzymatic velocity, Michaelis-Menten kinetic parameters, and mechanism of enzymatic inhibition (Medintz et al. 2006). Lastly quantum dots have been used in the development of multiplexed detection and imaging due to their broad absorption band and tunable emission wavelength dependant on particle size (Chan et al. 2002).

1.3.3 Gold Nanoparticles

Nanostructures made from noble metals, such as gold, have a unique photoactive effect. Monodispersed gold nanoparticles around 20 nm present a characteristic surface plasmon mode absorbance at 520-530nm which can be exploited in optical detection systems (Bellino et al. 2004). These gold nanoparticles have strong, size-dependent optical properties (Link and El-Sayed 2000). Gold self-assembled-monolayers (SAM) have previously been used to enhance fluorescence on surfaces of many sensing substrates (Aslan et al. 2005; Haes et al. 2004). Additionally, gold nanoparticles have been found to act as fluorescent quenchers and are being used both in solution and on solid substrates (Aslan and Perez-Luna 2004; Nikoobakht et al. 2002; Wink et al. 1997). Conjugating to the surface of gold nanoparticles has proved to be very easy utilizing a covalent link between gold and free sulfhydryl groups (dative binding) as explained in

Bioconjugate Techniques (Hermanson 1996). Lastly, gold nanoparticles, like quantum dots, when their size drops below the Bohr radius (<1nm) they can also fluoresce at different wavelengths depending on how many atoms exist (Link et al. 2002; Zheng et al. 2004).

Advantageous of gold nanoparticles include their biocompatibility and ease of production. Gold nanoparticles have previously been utilized in many sensing applications including surface plasmon resonance (SPR), color changing assays and fluorescence enhancing and quenching analysis. The detection principle of SPR is based on changes is refractive index of a sample and is probably the most common method using gold nanoparticles (Nath and Chilkoti 2002). For instance Mateo-Marti developed a DNA biosensor on gold surfaces with peptide nucleic acids self-assembled monolayers (SAMs) and found them capable of hybridizing complementary DNA (Mateo-Marti et al. 2007). Applications in diagnosis and medical imaging using colloidal gold nanoparticles have been realized since the early 1980s, but only recently have taken off with the emergence of nanotechnology. A review by Sonvico sums up many of the new developments for metallic nanoparticles for detection, identification and quantification as well as for therapy applications (Sonvico et al. 2005). Another group combined gold nanoparticles as fluorescent quenchers with aptamer probe for protein detection (Wang et al. 2008). Lastly colorimetric sensing has been realized using aggregating gold nanoparticles to study biomolecular interactions (Basu et al. 2008; Nath and Chilkoti 2002). One known problem with gold nanoparticles in solution is the resulting aggregation that can occur if solution requirements are not met. Gold colloidal solutions

can be easily disrupted with adding biomolecules and changes in pH (Olofsson et al. 2003).

1.3.4 Nanofibers

The utilization of nanofibers as a matrix material is advantageous to many applications including tissue scaffolds and sensing platforms (Aussawasathien et al. 2005; Ma et al. 2005; Wutticharoenmongkol et al. 2006). Nanofibers consist of polymer or glass material and can be produced by electrospinning. Electrospinning is a technique to produce non-woven nanofibers using a high electric field applied to solution and driven to the counter electrode (Agarwal et al. 2008; Lee et al. 2007). Polymer nanofibers provide a high degree of flexibility for the sensor surface to interact and a transparent interface for optical signal detection. The surface is ideal for immobilization of biorecognition molecules. Nanofibers allow for diffusion of a sample solution or gas to permeate through the sensing surface (Sadek et al. 2007). While the pores can be made large enough for the lower molecular weight molecules to flow through, higher molecular weight molecules that might interfere with the detection of the analytes can be prevented from entering the matrix. According to Wang, nanofibrous membrane sensors have shown an order of magnitude higher sensitivity to 2,4-dinitro toluene than for sensors built on continuous films (Wang et al. 2002). Another group demonstrated a nanofibrous sensor for ammonia gas in the limit of 300 ppm (Manesh et al. 2007).

Nanofibers as sensing platforms must be optimized to maximize surface area per volume and modified for immobilization of biorecognition molecules. Investigations of solution properties and electrospinning parameters have been completed for a number of

potential polymer materials. The surface morphology of the nanofibers is of great importance and has been studied to produce thin, uniform nanofiber sensing platforms (Eda and Shivkumar 2007; Koski et al. 2004; Lin et al. 2006; Son et al. 2005).

The nanomaterials described have demonstrated great potential as fluorescent platforms for biosensing applications. Each material has its advantages and disadvantages, which can be chosen based on the specific application.

1.4 Characterization

Biosensors are characterized with many different criteria, such as sensitivity, selectivity, and response time. Sensitivity is usually the most important criterion and is defined as the measure of how great of a change in sensor output due to analyte interaction (Ligler et al. 2002). Selectivity deals with the accuracy at which the target analtye interacts with the sensor and response time with the time required to detect and observable and measurable signal. Optical detection data is usually taken in wavelengths versus intensity and can be analyzed find time response and dose-response curves. From these we can obtain the limit of detection and the response time.

Many pieces of equipment are used to characterize the performance criteria of biosensors. For fluorescence biosensors, a spectrofluorometer is often used. Spectrofluorometers consist of an excitation source, a sample chamber, and a detector often a photo multiplier tube (PMT) along with a series of mirrors and lenses to excite and then collect fluorescence signals. A UV-Vis spectrometer is often used to collect absorption data so that the correct excitation wavelength will be utilized for fluorescent measurements. Additionally, microscopy can be utilized to either physically view the

surface a sensing platform or could be adapted to collect fluorescence. Lastly, optical benches can be set up with individual components, such as filters, lenses, and detectors, to acquire the sensor signals.

CHAPTER 2

INTRODUCTION

2.1 Medically Relevant Enzymes

A novel optical biosensor technique has been developed for protease detection utilizing nanomaterials and peptide substrates. Proteases are a class of enzymes that control many body functions. For example, thrombin is an enzyme of the serine protease type, and is an important pathophysiological factor in many cardiovascular diseases (Jaffer et al. 2002; Tung et al. 2002). Thrombosis formation is due to thrombin's role in cutting fibrinogen into fibrin producing a blood clot (Carr and Martin 2004; Dacres et al. 2008; Puckett et al. 2005). Controlling blood coagulation levels is of great public concern due to the increase risks of heart disease patients and the potential for death if a blockage occurs. Previous studies monitoring cleavage of thrombin substrates have been demonstrated (Dacres et al. 2008; Grant et al. 2004a; Guarise et al. 2006; Tung et al. 2002). In most of our experiments we have used trypsin for comparison studies of thrombin because it is readily available and at a much reduced cost (Takasaki et al. 1975; Whittaker et al. 1994).

Sensitive, accurate, and timely detection of thrombin could significantly increase healthcare for patients suffering from blood coagulating issues. Patients with heart disease, an aneurism, or post surgical complications could greatly benefit from this biosensor. Current methods rely on flow-based imaging procedures using computed tomography angiography. Time consuming laboratory test or guess work based on

observations and other vitals can be eliminated with a point of care device to detect this important protease. Newer methods of detection rely on the enzymatic work potential of thrombin and allow for assessing a patients coagulation capacity (Baglin 2005).

Under normal conditions, thrombin levels are very low (~1nM), but concentrations rise rapidly due to injury from the body's natural response (greater than 5nM) (Wolberg 2007). Lack of a fast and easy method to determine thrombin level has lead to the initiation of this research. The aim of this research was to examine an optical technique for developing protease biosensor utilizing labeled-peptide substrates on nanomaterials surfaces. By being able to detect thrombin, a doctor can be able to detect proper blood circulation and have the ability to optimize drug therapies as needed and would be useful both during and after surgical operations to indicate the onset of thrombus formation (Baglin 2005; Carr et al. 2003).

2.2 Detection Methods

Developments in sensing mechanisms and the application of nanomaterials for biosensing have lead to a new generation of sensor systems (Liang et al. 2005). The detection method utilized here is based on peptide substrates that are designed to cleave in the presence of the target analyte. The feasibility of the peptide substrate technique was previously demonstrated with free floating peptides prior to our investigation on nanomaterial platforms (Grant et al. 2004a). This technique just utilized a dual-label peptide in solution to detect thrombin. Although other fluorescent methods of detection for protease activity have been used like enzymatic and immunoassays, cleavage substrates are considered the most predominant method (Mahmoud et al. 2008). The

detection method utilized here uses FRET, which is a distance-dependent chemical transduction method and requires two fluorescent molecules termed the donor and the acceptor (Grant et al. 2005a). When the donor and the acceptor are in close proximity, energy can transfer from an excited state donor molecule to the acceptor molecule. The excited acceptor can then relax releasing this energy as fluorescence emission. A schematic of this mechanism is shown in Figure 2.1.

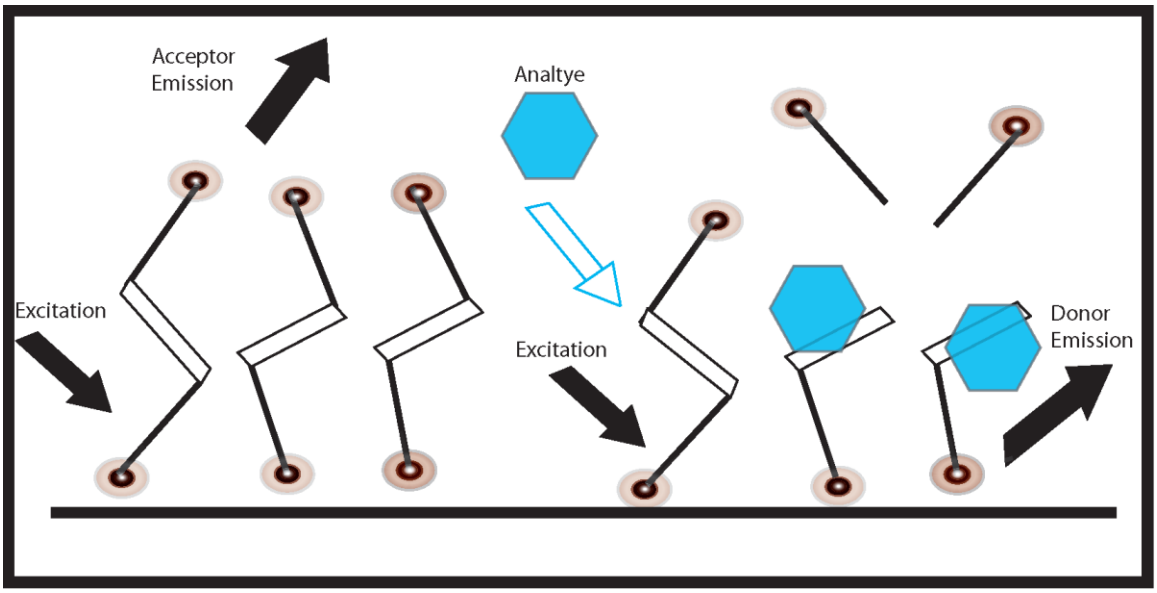

Figure 2.1 - Schematic of Peptide Substrate-based Biosensor

This technique has been leveraged to detect conformational changes of a peptide substrate that contains fluorophores on either side of the substrate and which contains an enzyme sensitive region that can be cleaved by a target analyte. In this case trypsin which cleaves the Arg-Gly bond is contained in the peptide sequence. Cleavage of the peptide substrate causes the fluorophores to separate in distance resulting in a fluorescence intensity change of the donor and acceptor signals. The donor signal that is

initially being transferred to the acceptor recovers as the distance between the fluorophores increase. The acceptor signal decreases as it is no longer able to absorb the energy from the donor molecule emission. The fluorescence emission spectra must be analyzed to determine whether the analyte is has acted on the peptide and this data can be further analyzed to determine the amount of analyte present by measuring the donor/acceptor (D/A ratio). This D/A ratio will increase with higher and higher activity of the protease and should remain the same with no protease.

Protease cleavage is used to monitor blood coagulation by measuring the FRET signal due to the dissociation of a peptide substrate. The association of thrombin with the substrate causes a conformational change that makes the cleave site accessible (Dacres et al. 2008). Proteolytic cleavage works like a proximity assay by adjustments in signal due to the distance between the fluorescent molecules. The thrombin specific cleavage sequence, Arg-Gly, was used in all peptide substrates designs. For FRET detection the ratio was calculated as emission ratio measured at 450nm/520nm and for quenching assay's the emergence of a fluorescence peak at 520nm was analyzed.

2.3 Research Objective

Proteases play key functions in many normal biological processes and it is sometimes necessary to monitor these enzymes. We have been working on protease detection using peptide substrates for a number of years and during this time have seen a number of other FRET-labeled peptide substrate assays being developed to detect trypsin/thrombin, botulinum neurotoxin, hepatitis C virus, and HIV protease (Sapsford et al. 2008a). The versatility of the sensor system can allow for any number of applications

to be discovered. Significant improvement of the detection sensitivity was achieved by the utilization of nanomaterials as sensor platforms with peptide substrates.

We report the development of novel nanomaterial biosensors for detection of protease. The advantage of this biosensor, which is based on nanomaterial platforms and peptides substrates, is that it is designed to detect proteases with a higher sensitivity and a faster response due to increased surface area of the nano-structured platforms. We could achieve lower limits of detection, which dropped from the micro-molar range to the pico-molar range. The potential future use for these sensing platforms has been shown.

CHAPTER 3

DEVELOPMENT OF A PROTEASE BIOSENSOR UTILIZING SILICA NANOBEADS

ABSTRACT

We have utilized silica nanoparticles as an optical platform for the development of a protease biosensor. The biosensor is based on the chemical transduction method, Fluorescence Resonance Energy Transfer, FRET, which requires the utilization of special molecular groups (donor and acceptor fluorophores), whose combined purpose is to fluoresce in the presence of a given analyte when subjected to an external photon source. The donor and acceptor fluorophores were covalently attached at either ends of peptide constructs that were synthesized using Fmoc (9-fluorenylmethoxycarbonyl) strategy, a method for solid-phase synthesis of peptides. Two different peptide substrates were synthesized, one positive construct which was cleaved by the enzyme, trypsin, and one negative construct. The peptides were then immobilized to the silica nanoparticles using two procedures: silanization, a method of covalent attachment, and simply physical adsorption. The peptide-immobilized nanoparticles were then exposed to trypsin and the change in fluorescence was determined. The results showed that the positive peptide constructs were cleaved upon exposure to trypsin, separating the distance between the two fluorophores, resulting in detectable changes in fluorescence. The covalently immobilized nanosensors demonstrated higher stability and sensitivity than the physically adsorbed nanosensors. The nanosensors demonstrated a limit of detection of 12.3 µg/ml with a response time within 2 minutes.

3.1 Introduction

While literature is inundated with sensor articles that utilize immobilized enzymes on electrodes and optical platforms to detect a range of analytes, there are far less articles that actually detect the presence of enzymes (Chouteau et al. 2005; Miscoria et al. 2005; Takhistov 2004). Enzymes are vital for proper functioning of living systems. They facilitate all processes (cellular processes as well as metabolic transformations) critical to life in micro-organisms, plants, animals, and humans. Because of their importance, proteases, such as thrombin, trypsin, and lipase, have been detected using protein microarrays, colorimetric assay, and fluorescence resonance energy transfer techniques (Bebrone et al. 2001; Millington et al. 1995; Rivoal et al. 2002; Zhu et al. 2000). For example, a novel protein microarray for activity-based detection of enzymes has been developed (Chen et al. 2003). The sensing mechanism utilizes fluorescently-labeled, mechanism-based inhibitors that can selectively label enzymes. The authors reported high-throughput detection of enzymes (phosphatases, cysteine proteases, and serine hydrolases). Another group of researchers developed a novel imaging technique to monitor *in vivo* protease activity in patients with rheumatoid arthritis (Wunder et al. 2004). Cathepsin B, a cysteine proteases, is involved in degrading particular structures and is highly up-regulated in rheumatoid arthritis. By monitoring the presence and activity of cathepsin B, effective treatment regimes can be developed. The probe's design consists of self-quenching fluorophores bound to a polymer that contains a poly-L-lysine backbone. The probe was injected into mice and fluorescence was activated by enzymes, such as cathepsin B, that cleaved the Lys-Lys site. Near-infrared fluorescence

(NIRF) imaging then determined the intensity of fluorescence at the joints of arthritic mice. Fluorescence in the toes and paws of the arthritic mice was significantly higher than that of non-arthritic mice. Another technique to detect enzymes involves resonance energy transfer. A thrombin optical biosensor that utilized fluorescence resonance energy transfer was developed by the authors (Grant et al. 2004a). Thrombin is a coagulation protein, which is an enzyme of the serine protease type, and it is an important pathophysiological factor in many cardiovascular diseases. Peptide constructs were synthesized with fluorophores conjugated at either. Upon cleavage by thrombin, the fluorophores are separated, resulting in a change in fluorescence. Sensitivity could be achieved to 0.02 units/ml.

While great strides have been made in biosensor technology and new sensing mechanisms are being developed, many technological problems remain, such as lack of real time monitoring and poor sensitivity. To improve sensor performance, nanosized materials, such as gold nanoparticles and silica nanoparticles, have been utilized as optical sensor platforms, while also many of these nanomaterials (gold nanoparticles and quantum dots) are utilized as photoactive nanomaterials (Liang et al. 2005; Olofsson et al. 2003; Qhobosheane et al. 2004; Sonvico et al. 2005). For example, a colorimetric gold nanoparticle biosensor was developed to study biomolecular interactions in real-time (Nath and Chilkoti 2002). Colloidal gold nanoparticles were self-assembled onto a glass substrate. As the analyte, streptavidin, bound to the gold, there was a detectable change in the absorbance spectrum. Another group of researchers utilized 250 nm magnetic nanoparticles (Graham et al. 2005). The nanoparticles were functionalized with

complementary and non-complementary target DNA. Magnetoresistive biochips were utilized to quickly focus and hybridize the nanoparticles. The method achieved an equivalent of 2-200 fmole/cm on the functionalized nanoparticles with complementary DNA.

We utilized silica nanobeads, 15 nanometers in diameter, as an optical platform for the protease biosensor. The protease biosensor is composed of a peptide construct labeled with fluorophores on either ends of the peptide. The goal of this study was to increase the surface area of the sensor platform for peptide immobilization and hence improve sensitivity. The silica nanobeads had a surface area of 42.2 nm^2, which would allow approximately 42 peptides/nanobeads, if the foot print for the peptide is approximately 1 nm^2. The peptide biosensor utilizes the chemical transduction method, Fluorescence Resonance Energy Transfer, FRET, which requires donor and acceptor fluorophores, whose combined purpose is to change fluorescence in the presence of a given analyte. As shown in Figure 3.1, the nanoprobes utilizing FRET switch their fluorescence wavelength between the donor, denoted by λ_1, and the acceptor, λ_2, dyes as distance between the two dyes change. When the peptide is cleaved by the enzyme, the donor and acceptor fluorophores are separated, resulting in a detectable change in fluorescence; i.e., an increase in donor fluorescence with a decrease in acceptors.

The above relationship is based on the phenomenon first described by Förster, where the proximity of molecules defined by their "Förster critical distance", R_0, is associated with equation below that defines the efficiency of FRET to be dependent on the inverse sixth power of the intermolecular separation, r (Lakowicz 1999).

$$E = R_0^6 / (R_0^6 + r^6)$$

This research describes the principle of operation as well as the method of production of a silica-based nanosensors. This novel technique to immobilize peptides on SiO_2 nanobeads has produced significant results.

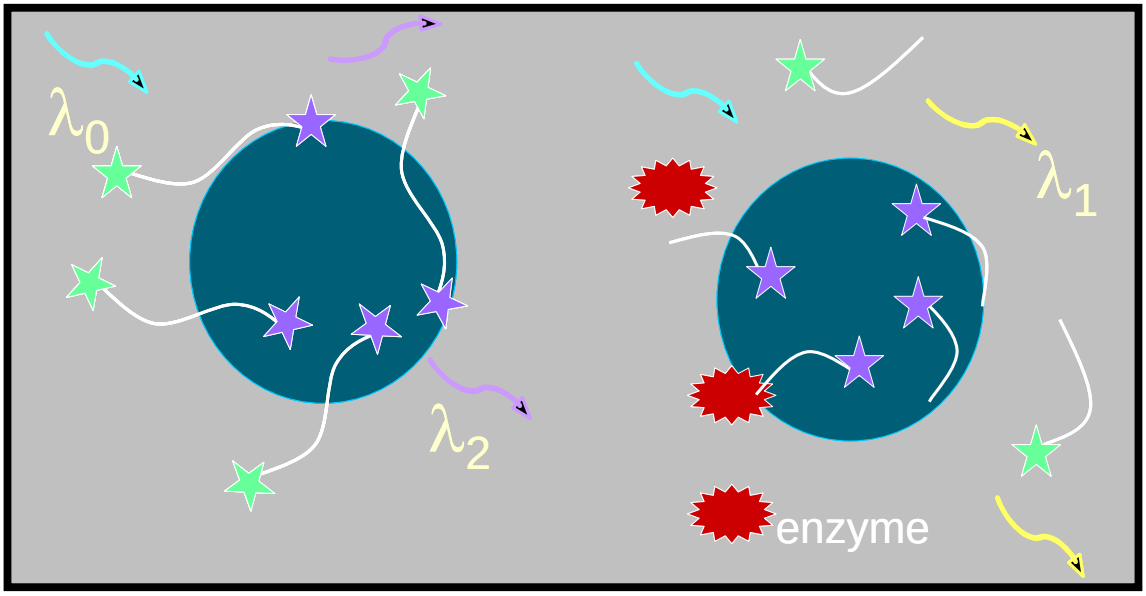

Figure 3.1 – Schematic of the Silica Nanoparticle Biosensor

3.2 Materials and Methods

3.2.1 Materials

Silicon dioxide nanopowder and the enzyme, trypsin, were purchased from Sigma-Aldrich (St. Louis, MO) as well as EtOH (Ethyl Alcohol) and PBS (phosphate buffered saline). MTS (3-Mercaptopropyl) trimethoxysilane and GMBS (N-succinimidyl-4-maleimidobutyrate) crosslinker were utilized as the silanization reagents

and were acquired from Fluka (Milwaukee, WI). DMF (Dimethylformamide) was also purchased from Fluka. Toluene was acquired from Fisher Scientific. The fluorophore, Fluorescein-(6)-carboxamidocaproic acid was used as the acceptor fluorophore and was purchased from Fluka. AMCA (7-amino-4-methylcourmarin-3-acetic acid) functioned as the donor fluorophore and was also purchased from Fluka.

3.2.2 Peptide Construct Synthesis

The synthesis of the peptide constructs are detailed in an earlier paper (Grant et al. 2004a). Briefly, the Fmoc (9-fluorenylmethoxycarbonyl) strategy, a method for solid-phase synthesis of peptides, was utilized. The two peptide sequences, CF6 and 51-4, were synthesized. The CF6 peptide construct contained five amino acids with an enzyme sensitive sequence (Proline-Arginine-Glycine). The 51-4 peptide was six amino acids long and lacked an Arginine in the enzyme sensitive region, making it the negative control. Table 3.1 displays the full sequence of the peptide constructs used in the experiments. The fluorescent fluorophore (Fluorescein-(6)-carboxamidocaproic acid) was coupled on the free ε-amino group on the lysine amino acid. The syntheses of each compound were then performed automatically with the corresponding sequence. After each sequence elongation, peptides on the resins were coupled with AMCA (7-amino-4-methyl-3-coumarinylacetic acid) to obtain the final desired sequences with two fluorescent labels.

Name	Sequence	Number of Amino Acids
CF6	AMCA-D-Phe-Pro-Arg-Gly-Lys-(Spacer-FL)-NH2	5
51-4	AMCA-Gly-Val-Pro-Gly-Val-Lys- (Spacer-FL)-NH2	6

Table 3.1 – Peptide Sequence for Silica Nanoparticles

3.2.3 Immobilization of Peptide Constructs to Silica Nanobeads

Following synthesis, the peptide constructs were immobilized onto silica nanobeads using two methods: silanization and simple absorption. Silanization is an important immobilization technique that has been utilized to attach biological agents to silica surfaces (Bhatia et al. 1989). Briefly, the procedure involves attaching a thiol-terminal silane film via the hydroxyl groups of the silica nanobead. This is following by a bifunctional crosslinker (such as GMBS, a heterobifunctional crosslinker) which will allow amide binding to the terminal amino group Lys. Specifically, approximately 65 mg of SiO_2 nanoparticles were suspended in 10 ml of 2% (v/v) MTS in toluene. The nanoparticles were sonicated to break up any clumping particles and puffed with nitrogen to ensure proper mixing. After a two hour incubation time, the mixture was then centrifuged for 10 minutes at 1100 RCF (3000 rpms; Hermle centrifuge). The supernatant was removed and the nanoparticles were rinsed with toluene. Again the mixture was centrifuged for 10 minutes at 1100 RCF and the supernatant was removed. The nanoparticles were allowed to dry overnight, with periodic puffs of nitrogen. Two mM of GMBS (in absolute ethanol) was added to the nanoparticles; sonicated for 1 hour and vortex every 15 minutes to it break up any clumps. After incubation, the mixture was

centrifuged for 10 minutes at 1100 RCF and the supernatant was removed. To the nanoparticles, 6.5 ml of PBS was added. The nanoparticle concentration was approximately 10 mg/ml. One ml of nanoparticle solution was aliquoted to 1.5 ml centrifuge vials and then 20 µg/ml of either CF6 or 51-4 peptide construct was added to each vial. The peptides were allowed to incubate at 4º C with constant mixing for at least one hour. After incubation, the vials were removes from the refrigerator and sonicated for 5 minutes at 1100 RCF. Again the supernatant was removed and the remaining nanoparticles were washed three times with 1 ml of PBS. The peptide-labeled nanoparticles were then resuspended in 1 ml of PBS until use. Approximately 20 µl of the mixture was removed, suspended in 2 ml of PBS, and characterized with an ISA FluoroMax-3 spectrofluorometer.

The peptide constructs were also simply adsorbed onto the silica nanoparticles. The procedure included adding 5.7 ml PBS to 0.065 g SiO_2 nanoparticles, in order to achieve a 10 mg/ml nanoparticle concentration. One ml of the nanoparticle solution was aliquoted in 1.5 ml centrifuge vials along with an amount of 20 µg of each of the peptides CF6 and 51-4. One vial was utilized as a blank control. The solutions incubated for 20 hours at 4°C with continuous stirring. After incubation, the solutions were sonicated for 5 minutes, then centrifuged for 5 minutes at 3300 RPM (1100 RCF). The supernatant was removed and saved. The remaining nanoparticles were vigorously washed 3 times with 1 ml PBS, using centrifuge and vortexing. Next 20 µl of the nanoparticle solution was removed, suspended in 2 ml of PBS, and characterized with the spectrofluorometer.

3.2.4 Response of the Constructs to Trypsin

After the peptide constructs were immobilized to the silica nanobeads, they were then exposed to trypsin and the time response and dose response were determined. The ratio of the donor intensity to the acceptor intensity (D/A) was analyzed at different time intervals and for various trypsin concentrations. The ISA FluoroMax spectrofluorometer was utilized to acquire the donor and acceptor fluorescence. The ratio was calculated as:

$$\text{AMCA/FL ratio (D/A)} = \frac{\bar{I}(\lambda = 445 \text{ to } 455nm)}{\bar{I}(\lambda = 515 \text{ to } 525nm)}$$

where $\bar{I}$ is the average fluorescence intensity for the donor and acceptor fluorophores.

3.2.5 Time Response of the Peptide Nanobeads

It was imperative to determine how fast the peptide nanobeads responded to the trypsin enzyme and to compare this response to peptide constructs not immobilized to nanobeads. For the time response experiments, approximately 20 µl of the peptide constructs (with and without nanobeads) were added to 2 ml of PBS and placed in microcuvettes. Time scans were performed upon the addition of 5 µl of trypsin (61.7 µg/ml). The solution was scanned at the following times: 0 (baseline), 10 seconds, 2, 4, 6, and 8 minutes after trypsin addition. The donor-to-acceptor ratio was calculated for each construct and plotted versus time.

3.2.6 Dose Response of the Peptide Nanobeads

For the dose response experiments, 20 µl of peptide nanobeads were suspended in 2 ml of PBS placed in 16 microcuvettes. A known concentration of trypsin was then

added to the cuvettes, stirred, and then immediately scanned with a spectrofluorometer, within 2 minutes. The concentration of trypsin utilized was 0, 1 µl, 5 µl, 10 µl, 15 µl, and 20 µl (12.3, 61.7,123.7, 185.6, 247.5 µg/ml). The change in fluorescence (donor/acceptor) was again recorded and plotted versus trypsin concentration.

3.3 Results

3.3.1 Immobilization of Peptide Constructs to Silica Nanobeads

Figures 3.2 and 3.3 display the spectroscopy scans of the nanobeads that were exposed to the CF6 peptide and the 51-4 peptide respectively. Each graph shows a spectrum of just the nanobeads (control solution) and spectra of the peptides in free solution, physically adsorbed, or covalently bound. The spectra of the control solution, which consist of nanoparticles without peptide immobilization, appeared flat in both graphs, as expected. The CF6 peptides that were silanized (covalently bound) onto the nanobeads displayed a higher and more defined fluorescence than the free CF6 peptides. These results indicate that the nanobeads are concentrating the peptides, which results in a higher signal. Additionally, the silanized nanobeads displayed a higher signal than the adsorbed nanobeads, indicating further bonding is occurring as well as stronger bonding and thus higher signals resulted with covalently bound peptides. It is interesting to note that the acceptor fluorescence peak is also nonexistent with the adsorbed CF6 peptide. Apparently, adsorption of the peptide disrupted the energy transfer, resulting in an absent acceptor peak. In all cases, it is noted that the fluorescent peaks of the bound peptides

(both covalently bound and physically adsorbed) were blue-shifted.

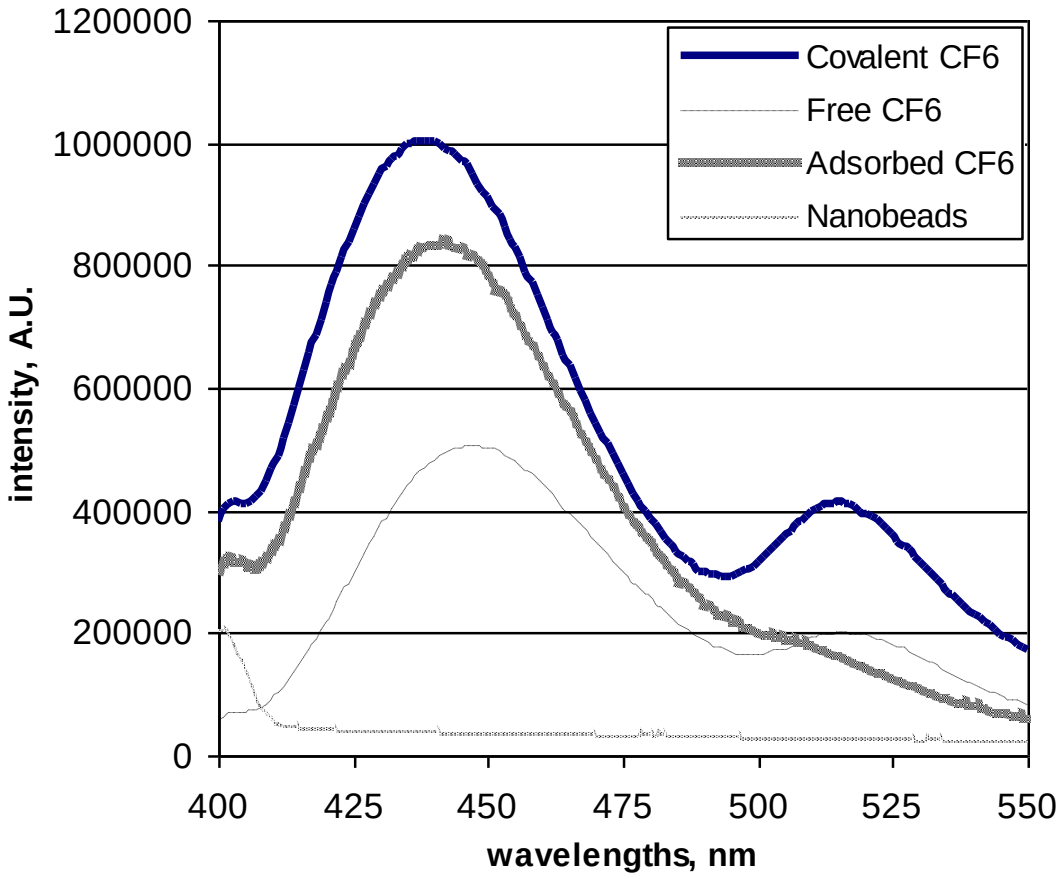

Figure 3.2 - Spectroscopy scans of the CF6 peptides that were in free solution, physically adsorbed, and covalently bound.

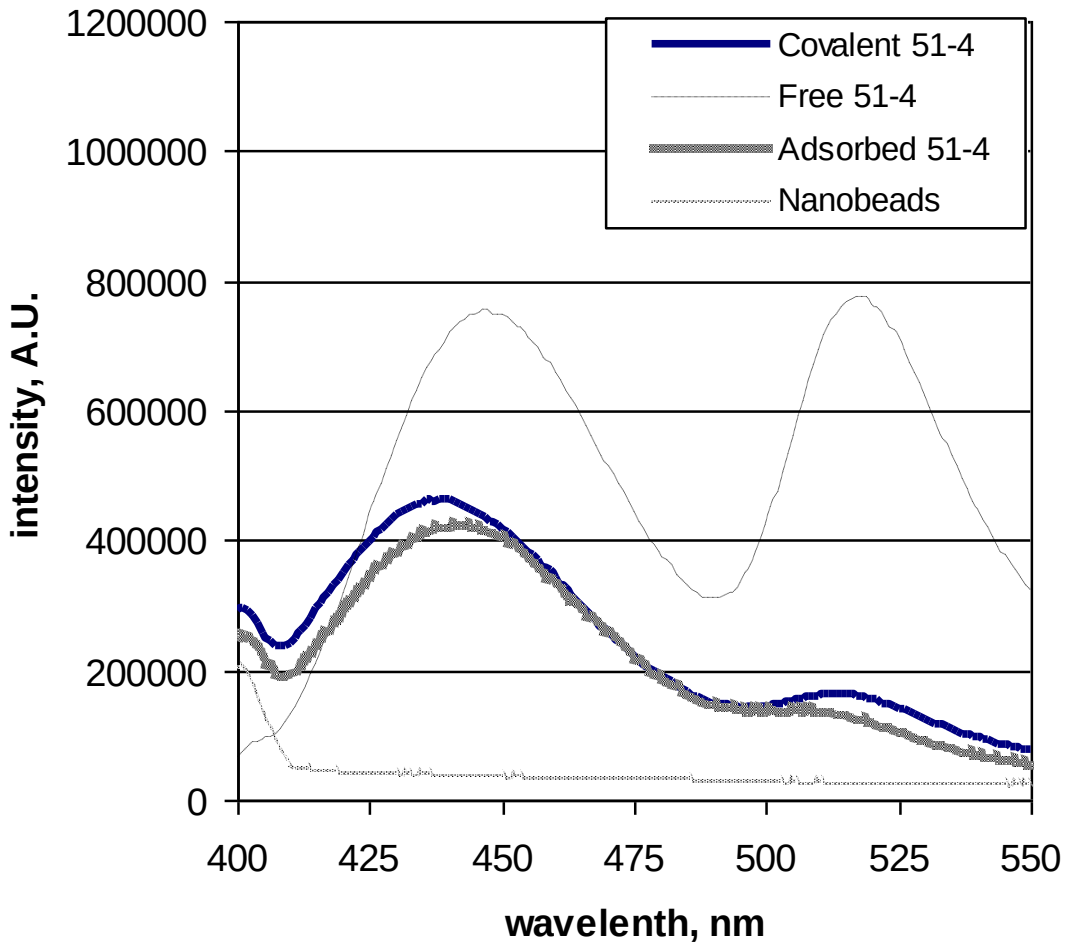

Figure 3.3 - Spectroscopy scans of the 51-4 peptides that were in free solution, physically adsorbed, and covalently bound.

The 51-4 peptide displayed some interesting results. The free solution peptide displayed very strong donor and acceptor peaks with a donor/acceptor intensity ratio of approximately 1. The covalent bound peptide displayed a smaller donor peak and a much smaller acceptor peak, with an intensity ratio of approximately 2.7. It is possible that the peptide underwent a conformational change upon binding, resulting in a change or disruption in energy transfer. The adsorbed peptides mimicked the covalently bound

peptides in a lesser scale indicating less binding, similar to the CF6 peptide. Again, a fluorescence blue-shifting of the bound peptide was noted.

3.3.2 Peptide Nanobeads Response Time

In Figure 3.4, the CF6 covalently bound peptides and CF6 adsorbed peptides were exposed to 61.7 µg/ml and the change in fluorescence was recorded over time. As time increased, the donor intensity peak (AMCA) peak increased, while the acceptor intensity peak (fluorescein) did slightly decrease. The experiments demonstrated the ability of trypsin to cleave the peptide at the enzyme sensitive region while immobilized onto a nanobead. The fluorophores were separated, resulting in a detectable change in fluorescence. Both the covalently immobilized and physically adsorbed peptides followed linear trendlines with an r-squared value of 0.976 for the covalently immobilized peptides ($y = 0.11x + 2.63$) and 0.940 for the physically adsorbed peptides ($y = 0.067 + 2.8$). The covalently bound peptides also demonstrated a faster response than the physically adsorbed peptides as indicated by the slope of the equations. Also, the 51-4 negative peptide was tested in free solution (no nanobead immobilization) and exposed to 61.7 µg/ml of trypsin. The results in Figure 3.4 showed that the 51-4 peptide was not cleaved by the enzyme, as noted by the little change in fluorescent ratio. Figure 4 also indicated that detectable changes in fluorescence occur within minutes of adding the enzyme and additional peptide constructs are cleaved as time progresses. Eventually, a saturation point will be reached when all of the peptide constructs are cleaved.

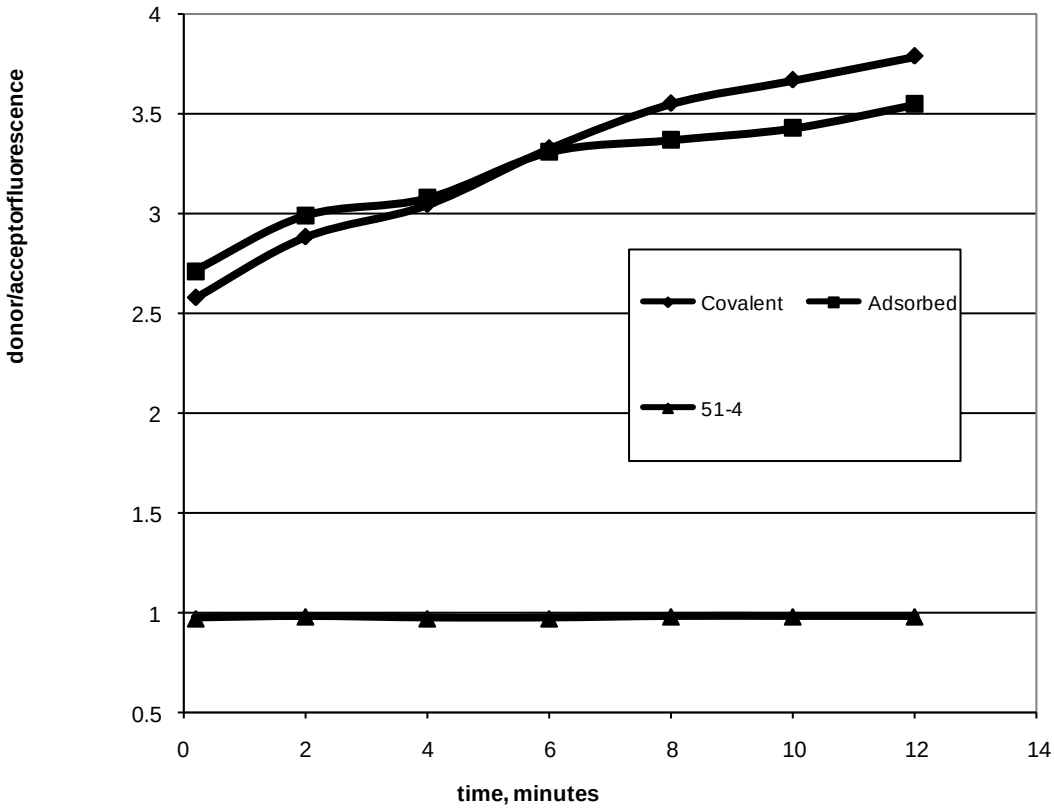

Figure 3.4 - Time response of the CF6 peptide covalently immobilized to a silica nanobead as well as physically adsorbed to a silica nanobead.

3.3.3 Peptide Nanobeads Dose Response

Figure 3.5 displays the dose response of the CF6 peptide silanized onto the silica nanobeads. The samples were prepared via silanization with a 2% MTS solution and followed by a 2nM GMBS crosslinker. The peptide was added and the samples were then washed and then scanned. Three trials of six different concentrations of trypsin were performed. The enzyme was given 2 minutes to cleave the peptide before scanning with the SPEX fluorometer. As shown in Figure 3.5, the response was significant at 12.3

µg/ml ($p \leq 0.0001$), 61.7 µg/ml ($p \leq 0.0006$), 123 µg/ml ($p \leq 0.001$), 185 µg/ml ($p \leq 0.002$), and 247 µg/ml ($p \leq 0.002$) of trypsin. The dose response curve appears to follow a rectangular hyperbolic curve which is indicative of the Michaelis-Menten equation, which describes substrate concentration on the initial rate of an enzyme-catalyzed reaction as shown in the equation below.

$$v = Vmax[S]/Km + [S]$$

where v is the rate of the reaction, [S] is the substrate concentration, and Km is the Michaelis-Menten constant. At low concentrations of trypsin, the availability of the peptide immobilized nanobeads is the rate limiting step. Thus as the amount of peptides increased, there appears to be a rapid increase in the initial rate of the reaction, i.e., the peptide is rapidly cleaved by trypsin. If more enzymes were added to the solution, there would be a saturation point where all the peptide constructs would have been cleaved. This would be the V_{max} of the Michaelis-Menten equation. Theoretically, Km could then be determined by calculating the Vmax/2 on the graph and interpreting the Km on the x-axis. Km is a guide of the affinity that trypsin has for the immobilized peptide constructs. Additionally from Figure 3.5, it was determined that the sensor could significantly detect 12.3 µg/ml trypsin in solution.

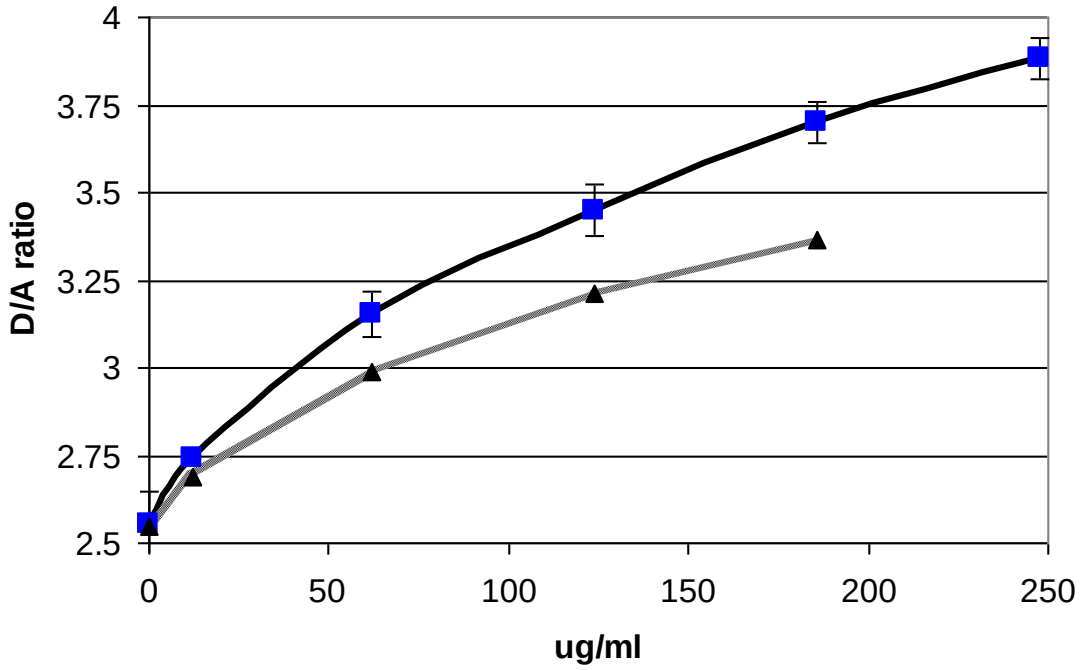

Figure 3.5 - Dosage response of the CF6 peptide silanized onto silica nanobeads.

We also tested the dose response of the CF6 peptide physically adsorbed onto the nanobeads. Three trials of six different concentrations were performed in order to determine if the concentration of trypsin has an effect on the immobilized peptide. Results were inclusive and insignificant with this method of immobilization compared to silanization. Sporadic and inconsistent responses were obtained. The physically adsorbed CF6 peptide did not results in any significant changes in fluorescence upon exposure to trypsin. Since physical adsorption is non-specific and non-directional, the peptide would have been randomly adsorbed onto the nanobeads. It is possible the peptide was rendered non-functional after adsorption i.e., the enzyme did not have access to the Arg-Gly enzyme sensitive region with many of the adsorbed peptide constructs.

3.3.4 Mechanism of Binding to the Nanobeads

It was desirable to ascertain whether the donor conjugated side of the peptide or the acceptor conjugated side of the peptide was binding to the nanobeads. This knowledge would permit tailoring the design of the sensor platforms. After the covalent CF6 nanobeads had been exposed to trypsin, the test solutions were placed in centrifuge vials. The vials were spun at 3300 RPM (1100 RCF) for 8 minutes. The supernatant was then extracted and placed in (1 ml) cuvettes and scanned with the spectrofluorometer. The results are shown in Figure 3.6. The graph displays the absence of the fluorescein peak and the presence of the AMCA peak, which indicates that the acceptor side of the peptide was immobilized to the nanobeads. The donor side of the peptide did not immobilize and therefore was cleaved from the nanobeads during the reaction and released into the surrounding liquid. Therefore the initial premise that the acceptor side allowed the amide binding to the terminal amino group Lys during silanization is most likely correct.

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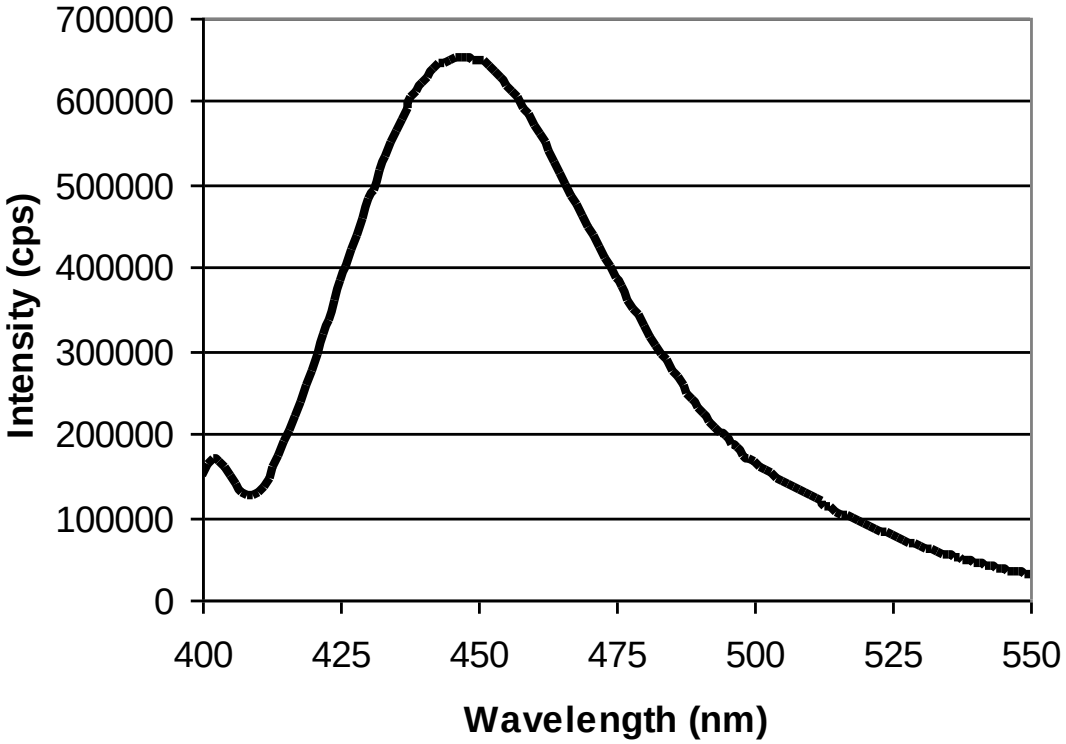

Figure 3.6 - Spectra that display the absence of the fluorescein peak and the presence of the donor peak, which indicates that the donor side of the peptide was immobilized to the nanobeads. The acceptor site of the peptide did not immobilized and was cleaved during the reaction.

3.4 Discussion

Silica nanobeads were utilized as optical platforms to immobilize peptide constructs for the detection of trypsin. Utilizing silica nanobeads offered the advantage of increased surface area in which to immobilize the peptide constructs while avoiding cross-reactions with free floating peptide constructs. The increased number of peptide constructs correlated to an increase in concentration which resulted in enhanced sensitivity.

In previous research, labeled peptide constructs were placed in phosphate buffered saline and the response of the sensor was recorded. These in-solution experiments inherently caused an increase in background signal. The donor from one peptide could interact with the acceptor on another peptide due to random, chance interactions between the free-floating peptide constructs. These random interactions could not be eliminated and was caused by Brownian motion or microthermal currents in the cuvette. Therefore it was imperative that the concentration of the peptide constructs remained low so as to avoid the cross-fluorescence. Immobilization of the peptide constructs onto the silica nanobeads offered an alternative: increased number of peptide constructs that could interact with the enzyme while avoiding cross-fluorescence. Immobilization onto silica nanobeads forced a particular arrangement of the peptide so that the acceptor side of the peptide was immobilized onto the nanobeads while the donor side was "free-floating". Any chance interactions between nanobeads would not translate into increase background signal since only the donor side of the peptide construct would be able to interact. Therefore, the concentration of the peptide constructs could be increased which correlated into enhanced sensor performance as noted by the lower detection limit. Additionally, the sensitivity of the immobilized peptide constructs was increased as indicated by the slopes of the dose response curves for the immobilized peptide versus the free floating peptides (Figure 3.5).

The utilization of nanobeads as sensor platforms could enhance sensor performance via the increase in the surface area for analyte-biosensing agent interaction. However, it is critical that the peptide constructs remain functional after immobilization.

The peptides were immobilized onto silica nanobeads using two methods: silanization and physical adsorption. The basic silanization process involved attaching a thiol-terminal silane film via the hydroxyl groups of the silica nanobead. This was followed by a bifunctional crosslinker which allowed amide binding to the terminal amino group Lys. For the physical adsorption, the procedure included incubating the peptides with the silica nanobeads. Covalent immobilization did not appear to disrupt the fluorescence spectrum of the CF6 peptide from its free solution structure. However, simple adsorption of the CF6 peptide did slightly disrupt the energy transfer to the acceptor, which resulted in a small acceptor fluorescence peak. For the 51-4 peptide, the free solution structure displayed a large acceptor peak while this peak is almost absent after immobilization. The 51-4 peptide had an extra amino group in its sequence that may have played a role in hindering energy transfer after immobilization.

The positive and negative peptides were constructed to have different lengths as noted in Table 3.1. The influence of the lengths to the Förster critical distance and the effectiveness of energy transfer were apparent in the Figures 3.2 and 3.3. Förster critical distance has been defined as the distance where the transfer of energy from the donor to acceptor is 50% efficient as stated in the equation 1 earlier.

The approximate Förster critical distance for the donor and acceptor pair of AMCA and Fluorescein is 52 Å. Therefore it was desirable to obtain an approximate labeling distance between the fluorophore pair on the peptide constructs that corresponded to $0.5R_o$-$2R_o$. When separation occurs after cleavage, then sufficient efficient energy transfer should occur. For the CF6 peptide construct, five amino acids

were utilized with the dyes conjugated at either ends. It was expected that the short separation distance would correlate to high energy transfer. However, as shown in Figure 3.2, the CF6 construct had a larger donor signal as compared to the acceptor signal. The inclusion of the arginine amino acid, which was necessary for trypsin cleavage, more than likely caused the peptide to form a 3D structure which hindered some of the energy transfer. The 51-4 construct was composed of 6 amino acids. The arginine group was not included in the sequence since cleavage by the enzymes was not needed. As noted in Figure 3.3, this peptide construct displayed equal donor and acceptor peaks. The structure was more conducive to energy transfer.

3.5 Conclusion

For the development of an enzymatic biosensor utilizing FRET, it was concluded that silica nanobeads can be used as an optical platform to immobilize peptides for the detection of trypsin. By increasing the surface area by using silica nanobeads we were able to get a fast response down to a limit of detection of 12.3 μg/ml (42.7 nM) trypsin in solution within 2 minutes. The results showed that the silanization method had increase in binding capability than simple adsorption. The adsorption method had poor initial fluorophore signal with little response to trypsin, most likely due to de-adsorption during the assay.

Acknowledgements

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Chapter 4

IMMOBILIZATION OF PEPTIDES ON QUANTUM DOTS FOR THE DEVELOPMENT OF A PROTEASE BIOSENSOR

Abstract

We have been developing a new optical biosensor platform for the detection of medically relevant enzymes. Two different peptide sequences have been synthesized as trypsin substrates that are designed to be immobilized onto the surface of functionalized quantum dot. The peptides were synthesized with the fluorophore, fluorescein attached to the opposite end of the peptides to allow for fluorescence resonance energy transfer (FRET) sensing. Sensors utilizing FRET switch their fluorescence wavelength between the donor (QD) and the acceptor dyes (Fluorescein) as distance between the two changes. When the peptide is cleaved by trypsin, the donor and acceptor fluorophores are separated, resulting in a detectable change in fluorescence.

The peptides were immobilized onto quantum dots using the EDC/s-NHS technique. Different molar ratios of peptide to quantum dot have been selected to determine efficient coupling. Quantum dots were utilized in order to increase the surface area for the peptide immobilization and because of the unique advantages of nanocrystalline particles. The substrates were then exposed to various concentrations of trypsin. Preliminary results showed that the trypsin cleaves the positive peptides resulting in a decrease in acceptor fluorescence with 42.7nM trypsin.

4.1 Introduction

Quantum dots offer a new optical biosensor platform for the immobilization of peptides in the detection of medically relevant enzymes. Quantum dots were utilized in order to increase the surface area for the peptide immobilization and for their enhanced fluorescent capabilities leading to improved sensitivity. Two different peptide sequences with only a single label have been synthesized as trypsin substrates that are designed to be immobilized onto the surface of functionalized quantum dots. The peptides contained the fluorophore, fluorescein, attached at the terminal end of the peptides to allow for fluorescence resonance energy transfer (FRET) sensing (Wu and Brand 1994). As mentioned, sensors utilizing FRET switch their fluorescence wavelength between the donor (quantum dot) and the acceptor (Fluorescein) as distance between the two changes (Grant et al. 2001). Quantum dots are being used as the donor fluorescent molecule. When the peptide is cleaved by trypsin, the donor and acceptor fluorophores are separated, resulting in a detectable change in fluorescence. Figure 4.1 shows a schematic of the technique. Different molar ratios of quantum dot to peptide were tested for optimization of the protocol and protein molecules were also used as a link between the quantum dot and the peptide.

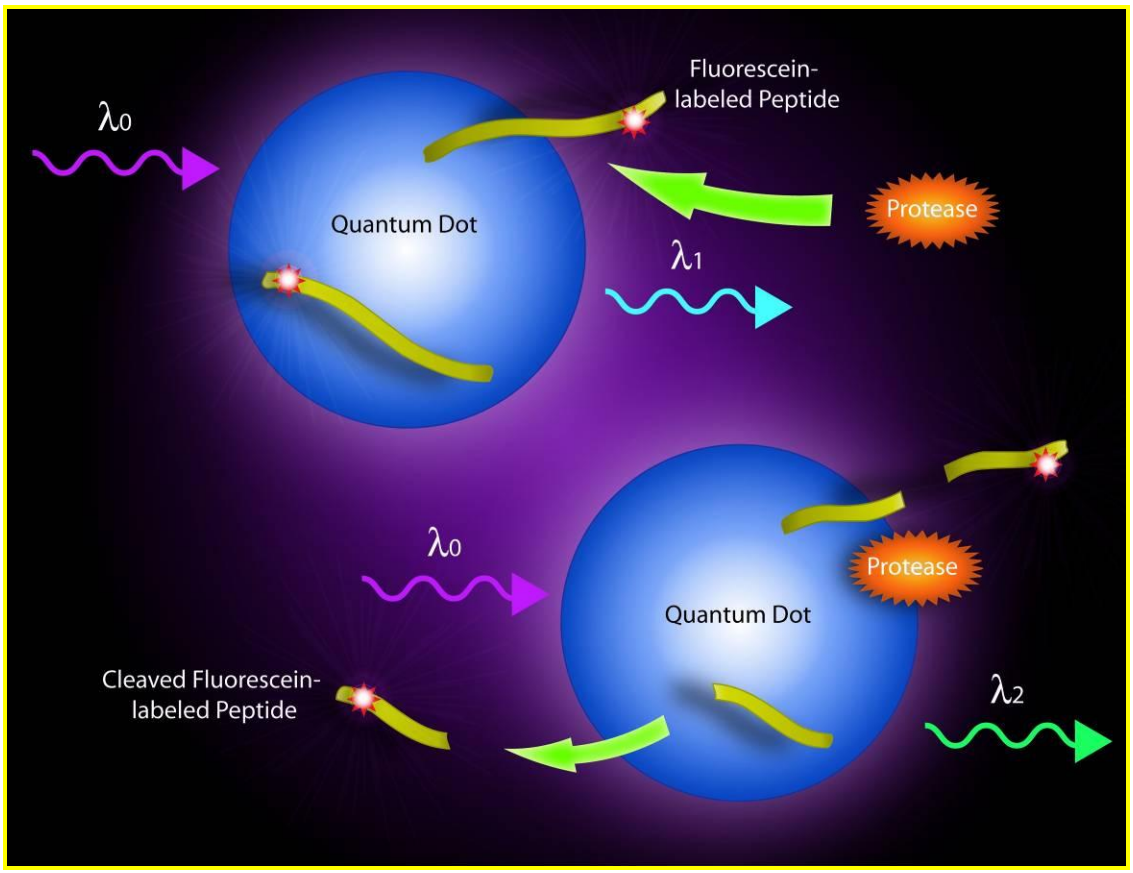

Figure 4.1 – Schematic of the Quantum Dot Biosensor.

The objective of this research was to investigate catalytic systems for the development of a biosensor that detects medically relevant enzymes. Although in order to control costs, trypsin was the enzyme utilized as the analyte, while, thrombin would ultimately be the enzyme we would want to detect. Thrombin is an enzyme found in the body as a result of an inflammatory response, as well as an important pathophysiological factor in many cardiovascular diseases (Ascenzi et al. 1982; Takasaki et al. 1975). Thrombin is a coagulation protein, which is an enzyme of the serine protease type that has many effects in the coagulation cascade, with its main role being to turn fibrinogen into fibrin, which in turn produces a blood clot. When thrombin is present in the blood

above 1nM concentrations, there is usually a pathological reason, which is the basis for needed to detect this enzyme.

The biosensor is based on the chemical transduction method, Fluorescence Resonance Energy Transfer (FRET), which is the radiation-less transfer of initially excited fluorescent donor molecule to a fluorescent acceptor molecule when the donor emission wavelength overlap with the acceptor absorption wavelength and when the molecules are within what is called a Forsters distance (Lakowicz 1999). The biosensor was designed to work on the principles of FRET where a quantum dot will be used as a donor molecule with a peptide substrate immobilized to its surface containing the fluorescent molecule, fluorescein at its terminal end. These types of sensors purpose is to fluoresce in the presence of a given analyte when subjected to an external photon source.

For fabrication of a biosensor on the nanoscale, quantum dots were utilized as an optical platform. Previously, we utilized silica nanoparticles and optical fibers as optical platforms. However, quantum dots were now used because of their enhanced optical properties. Quantum dots are semiconducting nanoparticles which have fluorescent properties due to quantum confinement (Medintz et al. 2005b). They come in a range of colors based on their size. Their enhanced properties over traditional fluorophores include increased brightness, narrow emission, high photostability and very long fluorescence lifetimes (>10ns) (Medintz et al. 2006).

The two peptide sequences, CF6 and 51-4, that were examined as a trypsin substrate shown in Table 4.1. The length of CF6 peptide construct contained 6 amino acids while the 51-4 peptide was 7 amino acids long. The 51-4 peptide was the negative

control which lacked the enzyme sensitive region Proline-Arginine-Glycine that is found in the positive peptide, CF6. The Proline-Arginine-glycine region is cleaved by the trypsin enzyme which will cause the distance between the donor and acceptor molecules to change.

Name	**Sequence**
CF6	H_2N-Cys-dPhe-Pro-Arg-Gly-Lys(Ahx-Fluorescein)-OH
51-4	H_2N-Cys-Gly-Val-Pro-Gly-Val-Lys(Ahx-Fluorescein)-OH

Table 4.1 – Amino Acid Sequence of Peptides for Quantum Dot Biosensor.

Quantum dots have been investigated as a fluorescent platform for the immobilization of peptide substrates for a FRET based assay. We hypothesize that the increased surface area per volume due to the nanosized particle will offer a fluorescent platform capable of transferring energy to the fluorophore on the terminal portion of the peptide. Trypsin will be added to the solution and a FRET change will occur due to the cleavage of the peptide substrate and the quantum dot (donor) and the fluorescein (acceptor) separating in distance. By using the quantum dot as a fluorescent platform it eliminates the need for multiple additional fluorophores attached at the binding site of the peptide substrate. Additional large molecules close to the surface of the platform and near the cleavage site could hinder access of the analyte in the sensing mechanism. Quantum dots have been found to be excellent donor in other applications and should enhance the sensitivity of our system (Clapp et al. 2004).

4.2 Materials and Methods

4.2.1 Chemicals and other materials

The quantum dots (Carboxyl T2 EviTags-Lake Placid Blue) were purchased from Evident Technologies (Troy, NY) and were made of CdSe core and a ZnS shell and contained a synthetic coating material with a functional carboxyl group for immobilization. EDC (1-ethly-3-[3-dimethylaminopropoly]carbodiimide) and s-NHS (Sulfo N-Hydroxysuccinimide) was purchased from Pierce Biotechnology (Rockford, IL). A 0.1 M MES activation buffer (pH 6.0) was made using MES hydrate and NaCl purchased from Sigma (St. Louis). 2-Mercaptoethanol, phosphate buffer saline (PBS), and bovine serum albumin (BSA) protein which was used to link the peptides and quantum dots as well was also purchased from Sigma. Molecular weight cut-off (MWCO) filters were purchased from Pall Corporation (East Hills, NY). Peptides were synthesized at the Structural Biology Core at the University of Missouri (Columbia, MO).

4.2.2 Peptide - quantum dot immobilization protocol

The quantum dots were conjugated to the peptide constructs using the EDC/s-NHS protocol which was supplied by the manufacturer. The quantum dots were initially activated by using EDC/s-NHS to form active ester on the EviTag surface. Briefly 0.4mg EDC (2mM) and 1.1mg Sulfo-NHS (5mM) were dissolved in 1ml MES activation buffer with 1ull of T2-EviTag quantum dots. This solution was incubated for 20mins at room temperature and gentle mixing. The reaction was quenched using 1.4µl (20mM) 2-Mercaptoethanol and incubated for 10mins at room temperature in a fume hood. Unreacted EDC/s-NHS was removed using a 100K MWCO spin filter and spinning at

10,000g (10,300rpm) for 5 mins. The peptide was then introduced to the activated quantum dots at different molar ratios (10:1, 5:1, 2:1, 1:1, and 1:5) of peptide to quantum dot in PBS. The peptides were allowed to incubate in the activated quantum dot solution for 2 hours with continuous stirring bonding at their accessible primary amine. After numerous washing steps with the spin filters, the peptide quantum dot complexes were formed.

Additionally protein molecules, BSA, were used to help link the peptides to the quantum dots. The peptides were first conjugated to a BSA protein molecule through a bioconjugation technique of attaching a thiol to amine functional group using SMCC (succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate) and reducing the BSA molecule using DTT (dithiotheritol). The BSA molecule containing the peptide molecule could then be attached to the quantum dot using the standard EDC/S-NHS protocol supplied by the manufacturer linking the carboxyl group on the quantum dot to the amine contained on the BSA protein molecule. This immobilization process was designed to fabricate the biosensor because in previous experiments direct immobilization of the peptide to the quantum dot resulted in decreased fluorescence from the quantum dot as a possible result of contact with DMSO (dimethyl sulfoxide), which was used to dissolve out the peptides. BSA was chosen as the protein molecule because of its mid-range size. Different molar ratios of peptide to quantum dot have been selected to determine efficient coupling. As mentioned before quantum dots were utilized in order to increase the surface area for the peptide immobilization and because of the unique advantages of nanocrystalline particles hoping to increase sensitivity of the biosensor. Protein

molecules are used to increase the forsters distance between the donor and acceptor fluorophore.

4.2.3 Peptide-Quantum Dot complexes characterization

The peptide-quantum dot complexes were initially scanned with a spectrofluorometer. Trypsin was then added in various amounts and the samples were scanned again investigating the time response and dosage response.

4.3 Results

4.3.1 Peptide-Quantum Dot response to trypsin

Figure 4.2 shows the results of the peptide-quantum dot being cleaved with trypsin at different time intervals. As the time increased, the acceptor peak decreased and shifted toward the donor peak. The results showed that it was possible to conjugate the peptides onto quantum dots and to cleave the peptide. This data taken of the CF6 positive peptide directly immobilized onto the quantum dot showed only a slight response but it indicated a decrease in acceptor signal which is the correct response we were looking for and also, it is noted that there is a slight blue shift in the peak. The sensor design gave a fast response but still needs optimization. The negative control peptide did not demonstrate any response to trypsin.

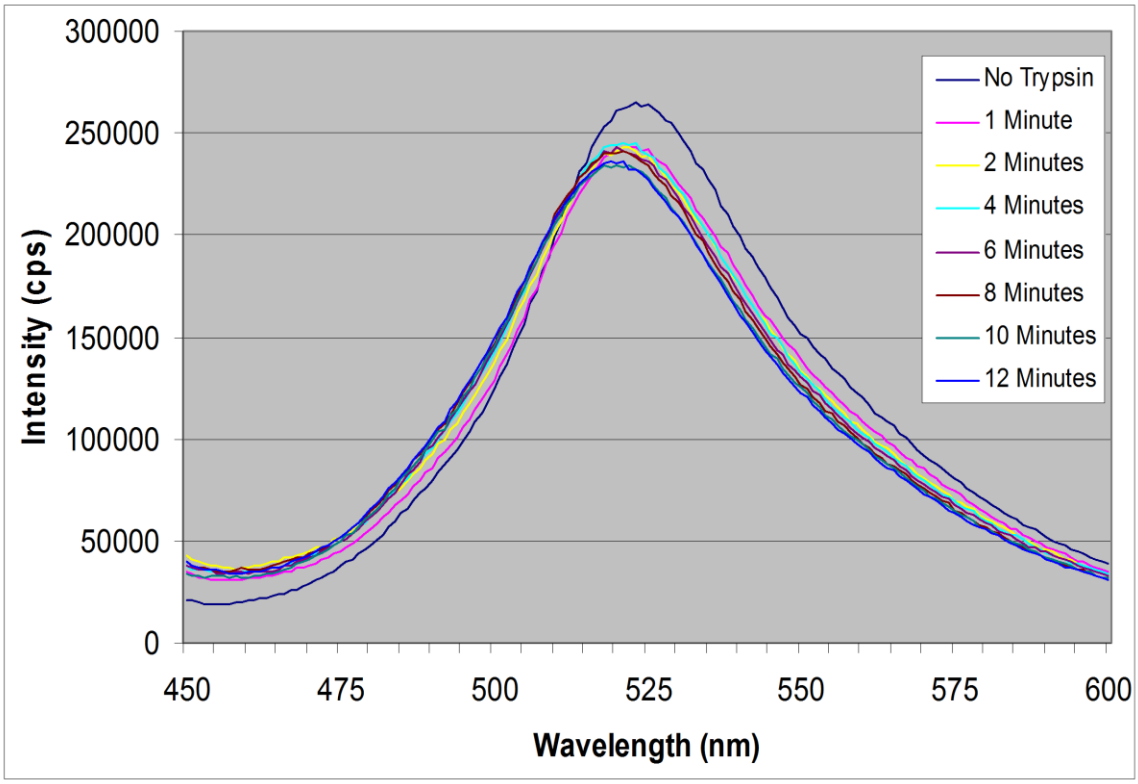

Figure 4.2 – Peptide-Quantum Dot response to trypsin.

4.3.2 Peptide-Protein-Quantum Dot response to trypsin

Results shown in Figure 4.3 are that of the peptide-quantum dot with the BSA, protein molecule. We see that as trypsin cleaves the positive peptides it results in a decrease in donor (quantum dot) fluorescence which was not expected. It was determined that due to the absence of the acceptor fluorescence signal that FRET is not taking place The sensor design gave a fast response but still needs optimization. The distance between the fluorophore on the peptide and the quantum dot could be too far or the quantum dot could just not be a feasible optical platform for this type of sensor. As expected the negative control peptide did not demonstrate any response to trypsin.

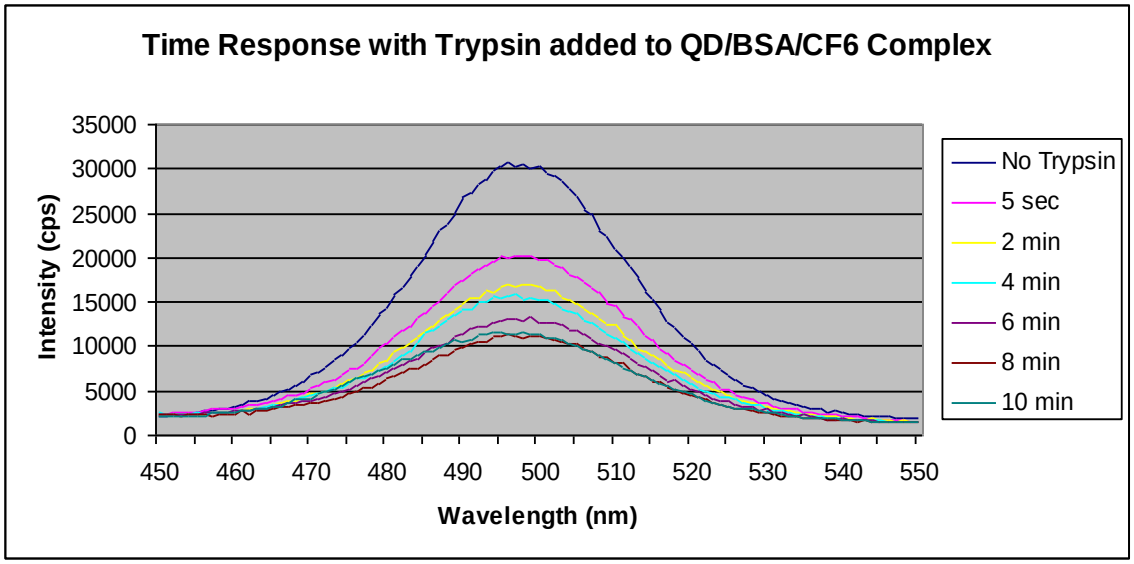

Figure 4.3 - Peptide-Protein-Quantum Dot response to trypsin.

4.4 Discussion

From the results it was found that although the QD/CF6 and QD/BSA/CF6 biosensor did show a response to trypsin over time that the quantum dots may be becoming unstable and deem them not feasible for this design for a protease biosensor.

4.5 Conclusion

For the development of a protease biosensor utilizing FRET, it was concluded that quantum dots can be utilize as an optical platform to conjugate peptides for the detection of trypsin but with poor results. Further research involved utilizing the single labeled peptides with gold nanoparticles acting as a quencher. Also, surface films of quantum dots and gold nanoparticles were explored.

Acknowledgements

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Chapter 5

DEVELOPMENT OF A PROTEASE BIOSENSOR UTILIZING GOLD NANOPARTICLE PEPTIDE CONSTRUCTS

Abstract

Gold nanoparticles have been investigated as a platform for immobilization of peptide constructs for the development of a biosensor to detect medically relevant enzymes, such as thrombin. Three different peptide constructs have been investigated as a protease biosensor. Two of the peptide constructs contain an enzyme sensitive region designed to be cleaved by the target analyte, but differing in amino acid sequence and length. The third peptide construct acts as a negative control, which lacks the cleavable region. Gold nanoparticles, 20 nm in diameter, were specifically immobilized to the n-terminal of the peptide construct via the strong interaction between gold and the amino acid cysteine. All of the peptide constructs were synthesized with a fluorophore attached at the c-terminal end of the peptide to allow for fluorescence sensing. The attached fluorescent molecule, fluorescein, will quench in the presence of the gold nanoparticle due to the plasmon resonance effect. When the peptide is cleaved by the enzyme, the fluorophore is separated, resulting in a detectable change in fluorescence intensity. The gold nanoparticle-peptide constructs, were tested in solution and their response to thrombin was recorded. The results showed that as the positive peptide constructs were cleaved by thrombin, a recovery in fluorescence occurred. The thrombin biosensor demonstrated a limit of detection down to 0.005 Units/ml with a response time within seconds.

5.1 Introduction

The unique properties of metallic materials have been leveraged in numerous sensing applications. Thin metallic films demonstrate surface plasmon resonance (SPR) effects and have been utilized as biosensors by detecting physiochemical changes in the films upon analyte interaction (Endo et al. 2005; Kawaguchi et al. 2007; Mateo-Marti et al. 2007). Additionally, gold nanoparticles in the range of a few nanometers demonstrate localized surface plasmon effects; thus, they are also being utilized in the design of biosensors (Link et al. 2002; Wang et al. 2008). Additionally, other studies have utilized quantum dots with gold nanoparticles as fluorescent quenchers with great success (Pons et al. 2007). As an example, amplified optical detection of thrombin in solution and on substrates has already been realized utilizing aptamer-functionalized gold nanoparticles as a catalytic label. Peptides were utilized with modified gold nanoparticles for cellular targeting to aid in the diagnosis and treatments of disease (Tkachenko et al. 2004). The optical properties of gold nanoparticles can greatly impact the biosensor industry thereby leading to new platforms for biological immobilization and fluorescent sensing.

Enhancements in biosensor performance have occurred via the development of improved sensing platforms and improved sensing mechanisms. Gold nanoparticles have been utilized to boost sensor signal performance (Zhang and Lakowicz 2007). Additionally, Hong et al. improved the optical signal (up to 10x) for a cardiac marker immunosensor by utilizing nanogold particles as fluorescent enhancers (Hong and Kang 2006). In a previous paper by (Grant et al. 2007), peptide constructs were immobilized to silica nanoparticles (20 nm in diameter) in order to increase the surface area for

attachment and thus improved the sensitivity of the biosensing technique. An order of magnitude improvement in the sensitivity was obtained for the detection of trypsin. This previous study was based on the ability of trypsin to cleave a peptide compromising a particular amino acid sequence resulting in a change in proximity between the donor and acceptor fluorescent molecules attached to either end of the peptide.

This chapter targets the development of a peptide-based biosensor to detect thrombin by utilizing gold nanoparticles to enhance the signal as well as providing increased surface area for binding. Thrombin belongs to the same family of trypsin proteases and similarly cleaves a specific amino acid sequence (Takasaki et al. 1975). Thrombin is a protease enzyme released in the body as a result of an inflammatory response; it is an important pathophysiological factor in many cardiovascular diseases, and it is involved in the coagulation cascade, with its main role being the conversion of the circulating plasma protein fibrinogen into fibrin, which in turn produces a thrombus (Puckett et al. 2005). By being able to detect the presence of thrombin more quickly and accurately, improvements in patient care can be obtained.

The objective of this research was to investigate the use of gold nanoparticles as fluorescent quenchers for a peptide-based biosensing design in the development of a point-of-care sensor to detect thrombin. We utilized gold nanoparticles, 20 nm in diameter, as a platform for the immobilization of the peptide constructs. The peptide constructs were attached via a functional thiol group contained on the amino acid, cysteine, on the end of the construct. As shown in Table 5.1, the positive peptide sequences contained an enzyme sensitive region (Arg-Gly) and a fluorophore on its distal

end. The peptides vary in the number and type of amino acids, thus changing its length, which affect its morphology. The negative peptide substrate lacks the specific enzyme cleavable sequence. When the peptide is intact, excitation of the fluorophore results in quenching of the emission of the signal. This is due to the close proximity (<5 nm) of the fluorophore and gold nanoparticle which absorbs strongly near 520 nm (Aslan and Perez-Luna 2004). Fluorescein was the fluorophore selected for the peptide substrate due to its emission overlap with the gold absorption. In the presence of a protease enzyme, the positive peptides will be cleaved by the enzymes and the cleaved peptide containing the fluorophore will be separated from the gold nanoparticles resulting in a recovery in fluorescence. A schematic of this sensor design is shown in Figure 5.1. We hypothesize that we can improve sensor sensitivity and achieve a fast response because the nano-size of the gold particles allows for increased number of peptide constructs binding to the gold nanoparticles (and thus the high surface area to volume ratio) and because of the increased interaction potential of the enzyme with the peptide constructs.

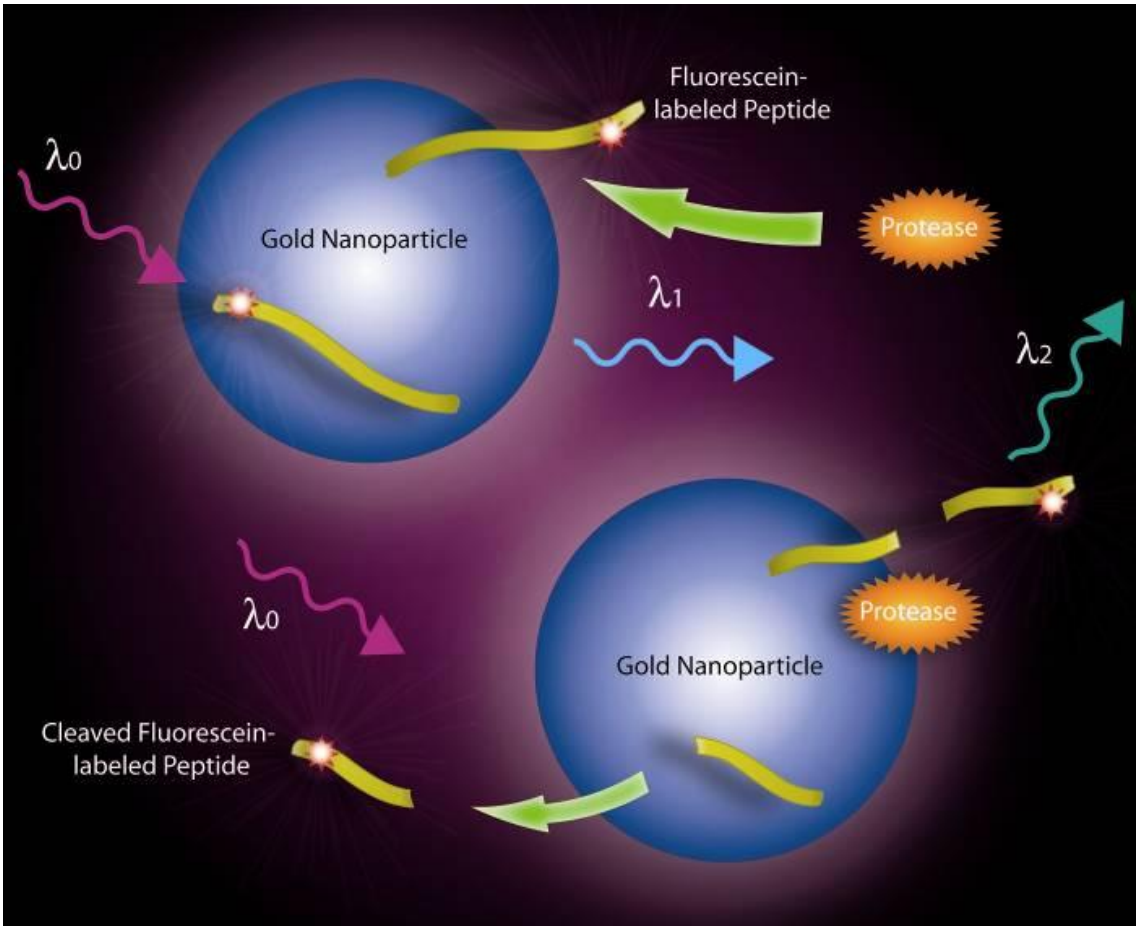

Figure 5.1 - Schematic of the gold nanoparticle immobilized with the peptide substrate. During excitation of the fluorescent molecule attached to the peptide, energy is transferred from the fluorophore and quenched by the gold nanoparticle. In the presence of trypsin, the peptide is cleaved resulting in a displacement of the peptide fragment containing the fluorophore moving a distance away from the gold nanoparticle resulting in recovery of fluorescence emission.

5.2 Materials and Methods

5.2.1 Chemicals and other materials

Gold nanoparticles (20 nm) were purchased from Ted Pella, Inc (Redding, CA). The particles are monodispersed in water ($7.0x10^{11}$ particles/ml) with only trace amounts of citrate tannic acid and potassium carbonate, leading to their net negative surface charge. Gold particles were characterized with a particle analyzer to ensure uniform size and

optically characterized with a UV-Vis (Beckman DU-520). The gold colloids particle size distribution was <15% (CV). Thrombin from human plasma was purchased from Sigma-Aldrich (St. Louis, MO). The fluorophore, 5(6)-carboxy-fluorescein, and phosphate buffer saline (PBS) was also purchased from Sigma-Aldrich (St. Louis, MO). The peptides were synthesized and purchased from the Structural Biology Core Facility at the University of Missouri (Columbia, MO).

5.2.2 Peptide synthesis

Three peptide sequences were synthesized as thrombin substrates in the design of the protease biosensor. The full sequences for CF6, 52-21, and 51-4 are shown in Table 5.1. The CF6 peptide construct contained 6 amino acids while the 52-21 peptide was 8 amino acids in length. These two peptides contain the (Arginine-Glycine) enzyme-sensitive region, differing only in length and spacer amino acids. The 51-4 peptide was the negative control which lacks the amino acid, Arginine, in the enzyme sensitive region and was 7 amino acids in length. The terminus of all substrates was a cysteine containing the thiol functional group for immobilization on the n-terminus and containing the fluorophore on the opposite c-terminus. The cleavable region is specific to the protease and will cause the substrate to separate between the arginine and glycine, releasing the portion of the peptide that contains fluorescein from the immobilized portion of the peptide in the presence of thrombin.

Name	Sequence
CF6	H_2N-Cys-dPhe-**Pro-Arg-Gly**-Lys(Ahx-Fluorescein)-OH
52-21	H_2N-Cys-Gly-Val-**Pro-Arg-Gly**-Val-Lys(Ahx-Fluorescein)-OH
51-4	H_2N-Cys-Gly-Val-Pro-Gly-Val-Lys(Ahx-Fluorescein)-OH

Table 5.1 - Amino acid sequence for the peptide constructs.

While the complete synthesis of the synthetic peptide constructs was documented in a previous paper (Grant et al. 2004a), briefly, the process involves the 9-fluorenylmethoxycarbonyl (Fmoc) strategy, a method of solid-phase synthesis for peptides, linking individual amino acids in a defined order in a continuous-flow method. The peptide sequence was designed with specific amino acids capable of providing rigidity to the substrate. Precise amino acid assembly was critical so that the functional conjugation group from the cysteine amino acid and the coupled fluorophore (5(6)-carboxy-fluorescein), attached to a 5-length spacer-arm off the lysine amino acid, attached to opposite terminal residues of the peptide. A high-performance liquid chromatography (HPLC) was run to purify the peptide substrates. Liquid chromatography-mass spectrometry (LC-MS) was also performed for mass analysis. Following synthesis, the peptides were characterized with a fluorescence spectrometer (ISA FluoroMax-3 spectrofluorometer) to verify fluorescence.

5.2.3 Immobilization of peptides to gold nanoparticles

The peptide substrates were immobilized to the gold nanoparticle surface using a simple chemical binding method of gold to free sulfhydryl groups (dative binding) via the

cysteine on the n-terminal of the peptide. This immobilization technique is based on the ability of the positively charged sulfur atoms to donate electrons to the gold atoms causing a strong Au-S bond (Bellino et al. 2004). The immobilization process must be optimized in order to retain the negative charge on the surface of the gold colloid which creates the repulsion and stabilization of the nanoparticles. Excessive immobilization of the gold colloid can cause the solution to settle out due to the collapse of net charge between the gold nanoparticles. The rinsing procedure consisting of centrifugation must also be critically controlled to minimize degradation of the stability of the gold nanoparticle. A UV-Vis spectrometer was utilized to measure the stability of the gold colloids during immobilization. Any aggregation would result in a decline and shift in absorbance.

In optimizing the conditions for conjugation of the peptides to the gold nanoparticles and in determining the proper peptide to gold ratio, a preliminary titration was performed via a modified protocol from Bioconjugate Techniques (Hermanson 1996). The gold colloid solution had a pH of 7.0; which is close to the isoelectric point of the peptides. First, 0.5 ml of gold colloid solution was added to five vials. Peptide constructs were added to the gold solution in increasing concentrations (0, 85, 170, 255, and 425 pM) to determine the optimal quantity of peptide constructs. The solution was allowed to conjugate for 5 minutes. UV/Vis spectroscopy as well as observed color changes, from red to violet or blue, was utilized to help identify the appropriate amount of peptide to use in order to avoid destabilization of the gold solution. The absorbance data is shown in Figure 5.2 and the color change is shown in Figure 5.3. From this

preliminary titration, it was determined that the 85 pM concentration of peptide constructs maintains stability of the gold nanoparticles.

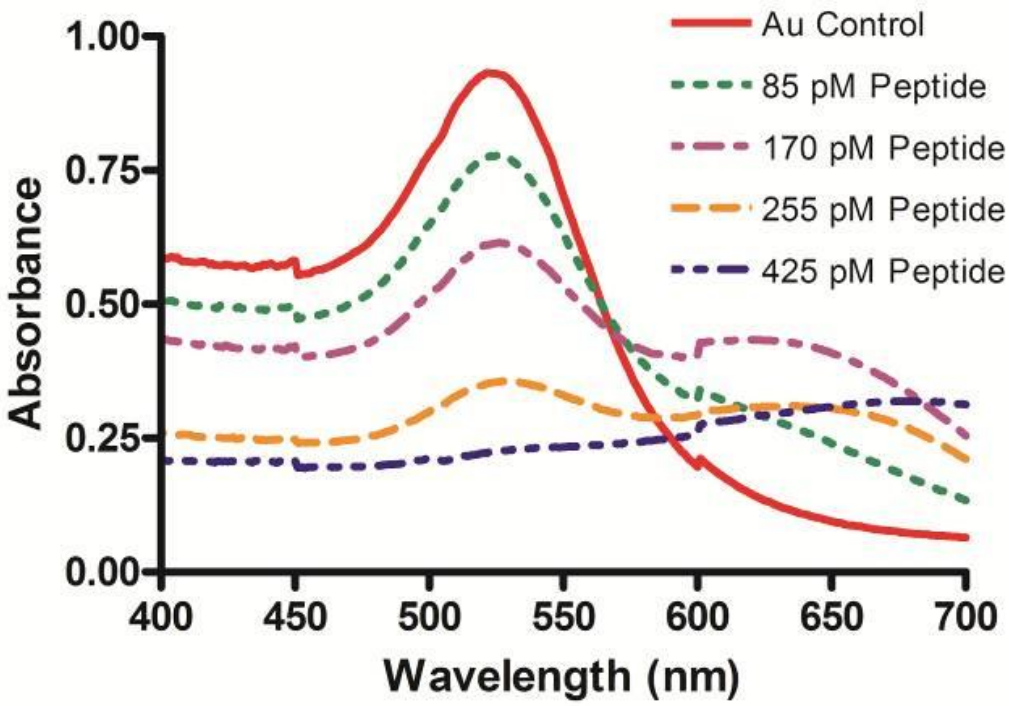

Figure 5.2 - UV-Vis absorbance data for gold nanoparticles immobilized with increasing concentrations of peptide.

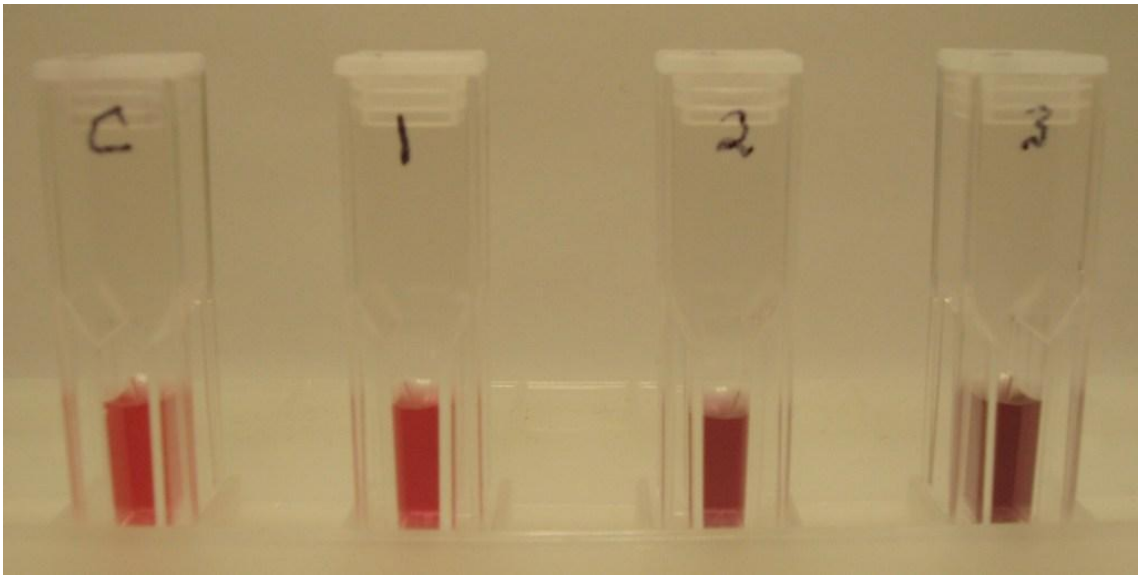

Figure 5.3 – Color change of Gold Solution with Increasing Amounts of Peptide.

Although using nanoparticles can increase the surface area for peptide binding, it is imperative that there is sufficient space for the enzyme to access the cleavage site on the particular peptide substrates. Additionally, electrolytes from surplus peptides can disrupt the negative repulsion effects between the gold particles in solution, resulting in the deeper color change due to the aggregation; thus, it is critical to remove all the surplus peptides. Also, saturating the nanoparticles with excess peptide may cause weakly bound substrates to detach contributing to higher background signals. The optimal peptide concentration to gold nanoparticle ratio provides the highest concentration of peptide substrate before gold nanoparticle aggregation occurs and thus optimizes the sensor response.

From the preliminary binding data, the immobilization protocol consisted of combining 0.5 ml of gold nanoparticle solution ($7x10^{11}$ particles/ml) with 85 pM peptide and conjugating for 30 minutes with continuous mixing. A washing step was performed

by centrifugation at 8,000 g for 1 hour at 4°C. The supernatant was removed and the pellet was resuspended in 0.5ml of deionized, distilled water. Again the mixture was washed by centrifugation using the same parameters and the supernatant was removed to ensure excess unbound peptides were rinsed away. The supernatant was then reconstituted into 1ml of water and was sonicated and vortexed to break up any clumps before use. The solution was characterized using a UV-Vis spectrometer to determine the absorbance of the gold bound with peptide, and fluorescence measurements were acquired with the spectrofluorometer to verify quenching between the gold nanoparticle and fluorophore terminated on the peptide.

5.2.4 Response of peptide constructs to enzymes

The gold nanoparticles immobilized with peptides were exposed to thrombin for time response and dosage response trials. For time response studies the enzyme was added to the sample in the amount of 0.02 Units/ml for thrombin and an initial scan was taken at about 20 seconds, followed by a 1 minute scan and then at every minute increment thereafter for 10 minutes. The ISA Fluoromax-3 was utilized to acquire the fluorescence intensity using an excitation wavelength of 494 nm and measuring the fluorescence signal at 520 nm. Five trials for each peptide sample were performed.

Dosage response experiments were also investigated where known concentrations from 0.005 to 0.08 Units/ml of the enzymes were exposed to the samples and a measurement was taken at 2 minutes. This data was then analyzed to present a concentration dependent recovery of fluorescence. Unless otherwise noted, all statistical analysis was carried out using GraphPad Prism version 4.0. A two-way ANOVA was

performed for a comparison test between the positive and negative substrates with significance set at $p<0.05$. Changes in dosage and time response curves were produced and standardized to detect the presence of the enzymes in the samples.

5.3 Results

5.3.1 Characterization of peptides immobilized to gold nanoparticles

An absorbance scan during the titration procedure of the gold nanoparticles was used to indicate the optimized concentration of peptides. The UV-Vis results for the 20 nm gold nanoparticles indicated a strong absorbance at 520 nm as shown in Figure 5.2. The stability of the gold nanoparticles continually collapsed with increased peptide immobilization thus resulting in a red shift in the absorbance scan. This was due to the loss of the negative net charge of the solution which is necessary for repulsion between the gold nanoparticles and thus keeping them in solution. The 85 pM amount of peptide solution was utilized as the optimized peptide concentration because the color change was minimal and the UV-Vis measurement indicated colloidal particles due to presence of the 520nm peak.

The peptide constructs were also scanned with the spectrofluorometer prior to immobilization and showed an emission peak at 520 nm, again due to the fluorophore. In order to demonstrate the quenching process taking place during introduction of the peptide to the gold nanoparticle solution, Figure 5.4 shows the spectrofluorometer scan of the gold nanoparticles prior to peptide immobilization as a flat line at the bottom of the graph. Figure 5.4 also shows the fluorescence increase and then decrease due to quenching occurring as the peptide is brought close to the gold nanoparticles due to

immobilization. The gold nanoparticle acts as a quencher due to its plasmon resonance property absorbing the 520 nm emission energy. For a more detailed description of the quenching phenomenon refer to Lakowicz's Principles of Fluorescence Spectroscopy (Lakowicz 1999). The CF6 peptide was selected to illustrate this phenomenon because it demonstrated the best overall response of the peptide constructs but all three substrates behaved in the same fashion. A sharp increase in fluorescence intensity is seen immediately after addition of CF6 peptide into the gold solution at 20 seconds and after each scan at 1 minute, 2 minute, 3 minute, and 5 minute the solution fluorescence intensity drops until it becomes saturated at about 5 minutes. Although this is still a considerable fluorescence signal this solution has not been put through a washing step yet and contains many unbound peptides. After washing occurs, the solution intensities drop to nearly that of the original gold solution indicating almost 100% quenching. This solution free of unbound peptides reduces background signal and allows for higher sensitivity and fast response of the sensor.

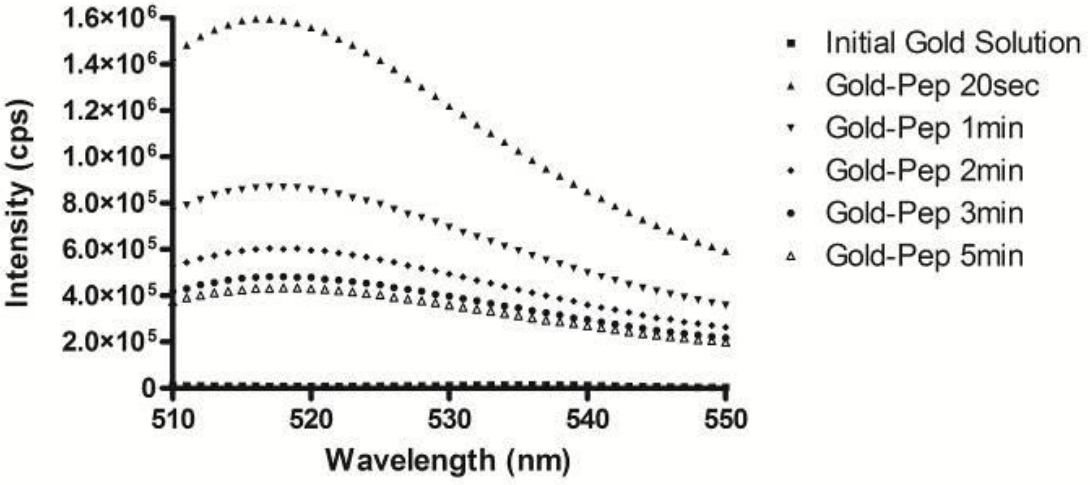

Figure 5.4 - Fluorescence quenching occurring due to CF6 peptide binding to gold nanoparticles over time.

5.3.2 Response of peptide constructs to enzyme

Time response and dosage response studies were obtained for thrombin. For the time response studies, as seen in Figure 5.5, the fluorescence emission was initially quenched before enzyme exposure. Immediately after the introduction of the enzyme, the fluorescence intensity was recovered. The fluorescence intensity continued to increase and then level off over time. It is noted that the fluorescence intensity level does increase for the negative peptide substrate time response data. This is most likely due to the fluorescence enhancement of the fluorophore due to a slight pH change with the addition of the enzyme. We see that over time the intensity increase from its initial spike stays constant with little increase. The data was processed and normalized to take into consideration the enhancements due to pH effects from the addition of the enzyme and the differences in the initial intensity levels of the peptide substrates. The results statistically indicated that there was a significant change ($p<0.001$) between the CF6

(positive) substrate and the 51-4 (negative) substrate with addition of thrombin, but statistically the difference between 52-21 (positive) and 51-4 (negative) is not significant ($p>0.05$). Since the 52-21 peptide substrate was two amino acids longer than CF6, the separation distance between the gold nanoparticle and fluorophore was too far and thus optimal energy transfer did not occur.

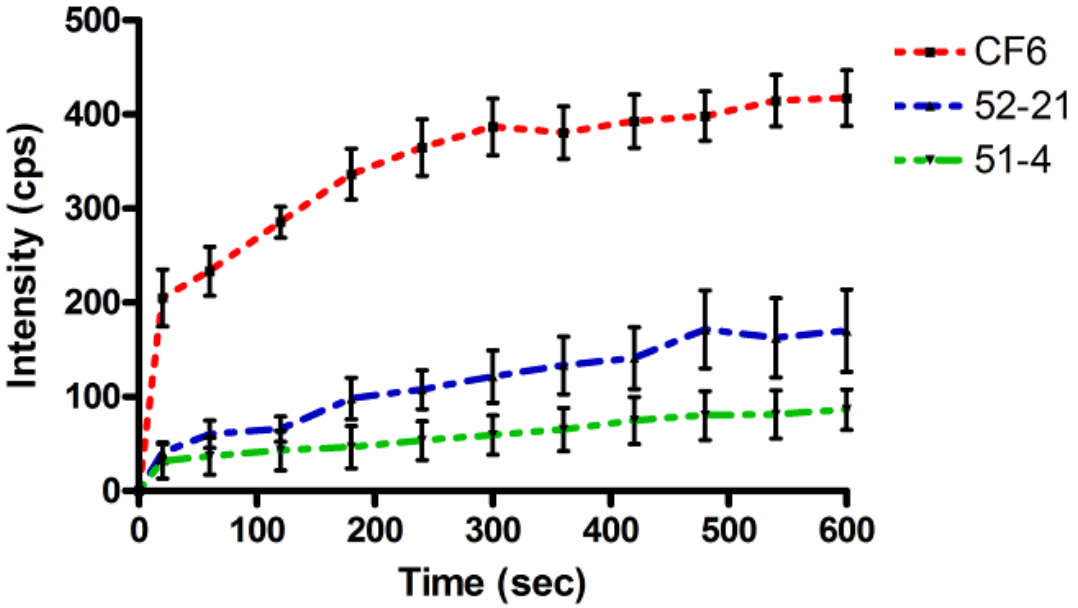

Figure 5.5 - Time response of gold/peptide substrates exposed to 0.02 Units/ml thrombin.

Results of the dosage response study of the CF6 peptide constructs are seen in Figure 5.6 for thrombin. As the dosage of enzymes are increased, cleavage of the peptide substrates increases indicated by higher fluorescence recovery signals at 2 minutes and the response continually increases and then levels off. There is a saturation level in which the reaction will not proceed any further regardless of increased enzyme concentration. The dosage response study indicated a limit of detection of 0.005 Units/ml with a near

immediate response time. The τ_{90} response time of the biosensors was within the first minute.

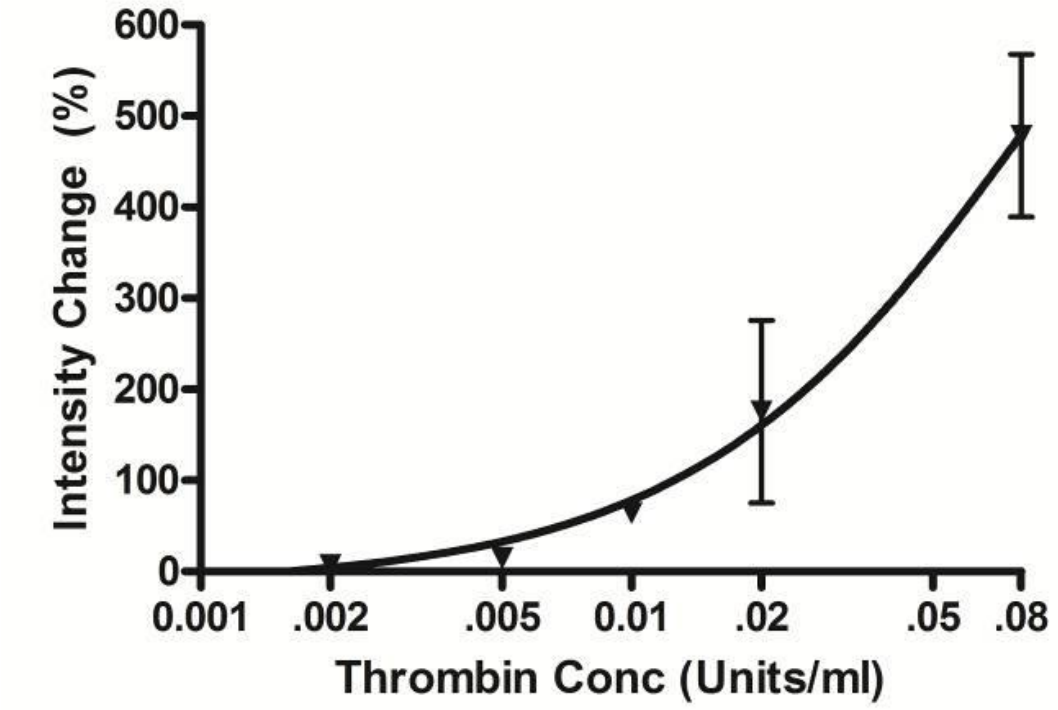

Figure 5.6 - Dosage response of gold/CF6 peptide exposed to different concentrations of thrombin after 2 minutes.

5.4 Discussion

Previous work by our group utilized a FRET-based system with a dual fluorescent-labeled peptide construct in solution. The use of gold nanoparticles substrates increased the sensitivity of thrombin from 0.02 Units/ml in our previous work to 0.005 Units/ml. This limit of detection is also significantly higher than the commercially available, Chromozym TH (Roche) assay kit, which has a limit of detection of 0.06

Units/ml. In the current chapter, using the gold nanoparticles as sensor platforms appears to enhance sensor performance over traditional organic dye FRET design.

We have utilized gold nanoparticles as fluorescent quenchers in a peptide-based biosensor for protease sensing. The gold nanoparticles have the unique property of exhibiting plasmon resonance and absorbance at 520 nm, making them ideally suited to complement the fluorescein labeled peptide substrate constructs. Also, due to the nanosized particles, we could achieve the added benefit of increased surface area of the platform for immobilization of the peptide and significantly increased sensitivity of the biosensor.

We experimentally determine the appropriate molar ratio of gold nanoparticles to peptide substrates by a simple titration experiment ensuring the functionality of the gold nanoparticle in solution as well as the peptide construct. An excess amount of peptide may cause leaching of weakly bound substrates and thus creating higher background signals. Conversely, utilizing less peptide could cause less intermolecular energy transfer after cleavage of the substrate which would lower the sensitivity.

The washing method also has proven to be an important step in the viability of the sensor because washing not only removes unbound peptide constructs, but also any fragmented sequences that do not contain terminal thiol groups for conjugation to the gold. The conjugation protocol utilized the strong interaction between the negative net charged gold colloids and the electron donating sulfur of the thiol group, which were specifically designed on the terminal end of the peptide in order to attach to the nanoparticle surface.

Our results showed that the pH effects needed to be considered (Yokoyama and Welchons 2007). The gold solution had an initial pH around 7, and both the enzyme activity and the fluorescence from the fluorophore are dependent on pH level. The addition of thrombin to a peptide solution caused a slight increase in the baseline of the fluorescence signal which was seen in the response of the negative peptide construct. Also the immobilization reaction should be carried out at or above the isoelectric point (~7.0) of the peptide constructs to promote optimized binding conditions.

Energy transfer between the coupled fluorophore and the gold nanoparticle is a distance-dependent occurrence and so the length of the peptide construct proved to be an important factor in the design of the sensor with regard to the number of amino acids (Saini et al. 2006), (Fan et al. 2005). The two positive peptide substrates and one negative peptide strand were synthesized with different amounts of amino acids in the sequence. The approximate distance between the gold nanoparticle and fluorophore of the positive CF6 peptide was determined to be about 36 Å and the positive 52-21 peptide was determined to be about 48 Å. The negative peptide 51-4 was estimated to be about 42 Å. The decreased distance between the fluorophore and the gold nanoparticle in the CF6 peptide resulted in stronger fluorescence quenching, resulting in a higher fluorescent recovery potential. Cleavage occurred in both the CF6 peptide and the 52-21 peptide, resulting in the release of a portion of the peptide containing the fluorophore. This increase in distance between the fluorophore and the gold nanoparticle resulted in a fluorescence recovery signal indicting a positive response to the enzyme. The negative

peptide strand showed only a small fluorescence recovery, which was predicted due to the absence of the enzyme cleavable region.

While peptides-based sensors have many advantages over antibody-based sensing systems in that they are smaller and more robust, a drawback of the peptide-based biosensor design is that it is not reusable (Soper et al. 2006). Once the peptide is cleaved by the target analyte the peptide is no longer usable.

5.5 Conclusion

Our results indicate that immobilizing peptides onto gold nanoparticles provides a viable method for protease detection. There was an immediate fluorescence recovery response upon addition of the enzymes to the biosensor. The dosage response study resulted in a limit of detection in the range of 0.005 Units/ml for thrombin with a response time within seconds. The speed at which the biosensor can determine the onset of thrombin formation has a profound use as point-of-care monitoring device. It has the potential to provide medical professionals insight into a patient's post-surgical health or response to drug therapies, which would be a major advancement over time consuming lab blood work. The peptide-based sensing mechanism coupled with the nanosized gold platform with a large surface area contributed to an enhancement in the sensitivity of the thrombin biosensor. Further work will involve incorporating the design into a lab-on-chip based device.

Acknowledgements

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Chapter 6

PMMA Nanofiber Sensing Platforms

Abstract

We are investigating the use of PMMA (polymethyl methacrylate) as a polymer material to electrospin into nanofiber sheets for flow-through sensing applications. The electrospun nanofibers offer advantages over planar solid surfaces due to their ability to interact with a potential analyte three-dimensionally. Nanofibers can serve as a platform for the attachment of biomolecules and present a higher surface area for increased attachment sites leading to higher signals compared to non-fibrous platforms. The development of nanofiber sensing surfaces requires optimization of the polymer solution and electrospinning parameters to achieve uniform, thin and functional fibers with increased surface area and flow-through capabilities. We have thoroughly characterized nanofiber platforms and chemically modified its surface for attachment of biorecognition molecules for biosensing applications. Additionally a flow through sensing design was developed using the modified nanofibers spun onto modified glass and PDMS (polydimethylsiloxane) wells. These nanofiber sensing platform achieve a 170% increase in signal potential over flat planar surfaces and vastly increase the surface area for interaction of the prospective analyte with the sensing surface. They have proven to be a viable system for sensing mechanisms.

6.1 Introduction

New advances in nanomaterials have led the way for improved biosensing methods. Nanoparticles have been heavily investigated as optical platforms for in-solution biosensing and nanoporous substrates are the benchmark for many solid substrate designs (Agarwal et al. 2008; Grant et al. 2007; Nath and Chilkoti 2002). These materials contain dramatically enhanced surface area over optical fibers and thin-films leading to increased sensitivity potential. The sensing platform serves as the interface between the recognition elements and the analyte interaction requiring an area with a high surface-to-volume ratio. There is also interest in developing a solid sensing platform that not only interacts in two-dimensions like a planar thin-film, but that is capable of sensing in three-dimensions. Nanofibers provide this ability by acting like a non-woven, mesh material that can be penetrated by the test sample. Some earlier research showed an order of magnitude higher sensitivity in nanofibers sensors than sensors formed from continuous thin films (Wang et al. 2002). These nanofibers can then further be made porous, offering not only a large area for interaction, but also an extremely high surface area-to-volume ratio (Manesh et al. 2008).

Nanofibers are produced by electrospinning, which is a simple, versatile technique to fabricate a non-woven, mesh material to be used as a sensing platform. A typical electrospinning apparatus set-up is shown in Figure 6.1 which utilizes a syringe pump, high voltage supply, and two electrodes. The fiber-forming solution is pumped from the syringe through tubing to a needle that is connected to the positive electrode of the high voltage supply. The collection plate is attached to the ground causing an electric

field between the electrodes. When the solution flows from the tip of the needle an electrically charged jet is induced pulling the fibers to the collection plate producing very thin fibers. The conical shape that forms, called the Taylor cone, is overcome by the electrostatic forces above a critical applied voltage ejecting the solution and proceeding to the collector (Frenot and Chronakis 2003). It has been described that the electrically charged solution is subjected to bending, whipping, splaying, or splitting motion caused by the mutual charge repulsion which causes the thinning (elongation) of the fibers as they reach the ground plate (Spasova et al. 2007). The solvent evaporates as the solution proceeds to the collector leaving solidified nanofibers. Some types of fibers also require additional processing after the nanofibers are formed. Further discussion will go into the properties of the electrospun solution and the controllable parameters of the electrospinning process that affect the morphology of the nanofibers. There has been much research on the benefit of producing thin fibers that provide a high surface-to-volume ratio. Reducing nanofiber diameter and inducing porosity has been shown to create large surface areas with good gas permeability compared to films (Manesh et al. 2008).

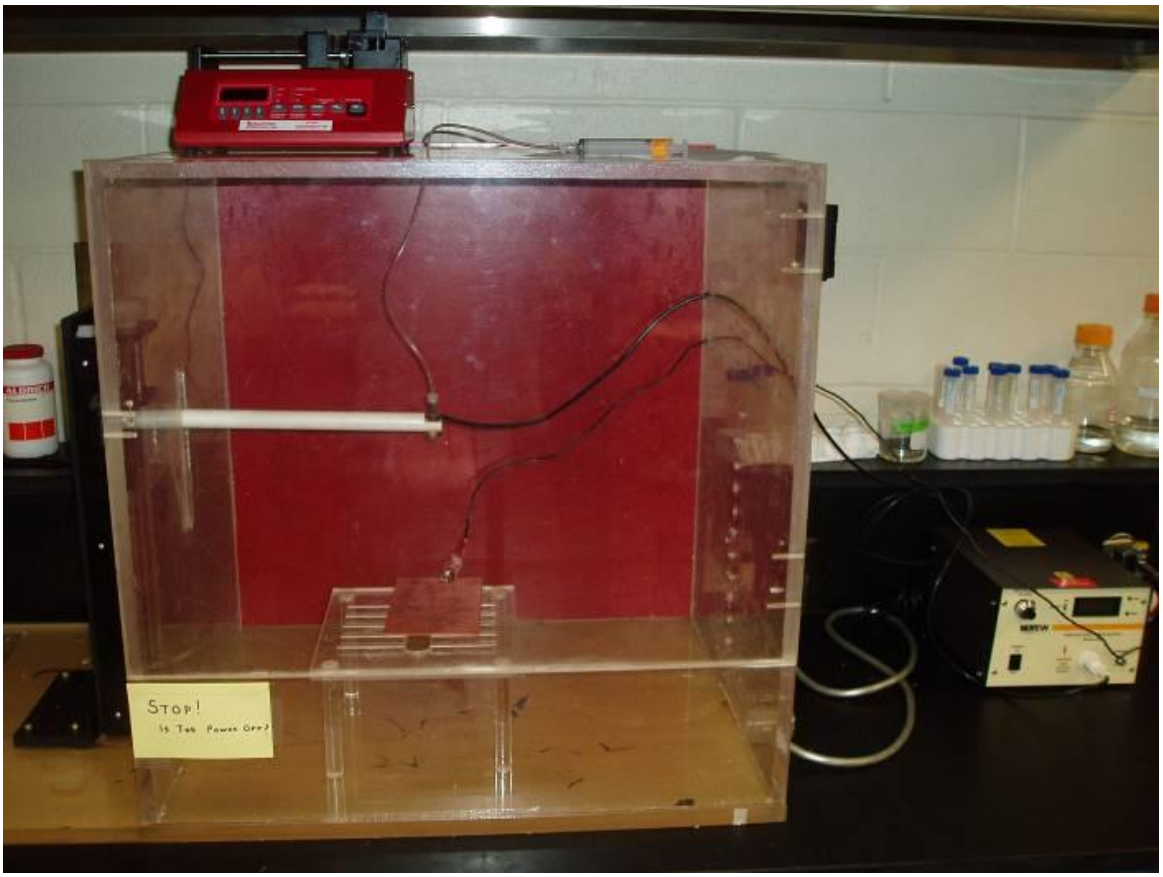

Figure 6.1 – Electrospinning Apparatus.

Fibrous porous nanomaterials are presently being utilized for many applications, including tissue engineering where researchers are utilizing a variety of electrospun materials such as collagen for scaffold development. In addition, nanofibers are being utilized in the design of biomedical devices such as sensors to improve sensor performance (Wang et al. 2002). For example, nanofibers have been used for the detection of high explosives where an air sample was preconcentrated and then flown through the nanofiber mesh. The injected trapped volume was allowed to interact with the sensing surface and then analyzed with detection limits in the parts per trillion by volume (pptv) of 2,4-di-nitro-toluene (DNT) (Tao et al. 2007). Another group utilized

thick layers of electrospun nanofibers using immobilized enzymes on gold electrodes for amperometric biosensing to measure glucose (Ren et al. 2006). Lastly, (Law and Tung 2008) also utilized peptides on hydrogel nanofibers for sensing of specific protease

We are electrospinning the synthetic polymer PMMA (polymethyl methacrylate) into nanofibers and utilizing the nanofibers as a sensing platform for peptide immobilization. PMMA is an optically transparent polymer, ideal for optical applications and contains methyl group side chains off its backbone that can be further modified to offer attachment sites for biologic recognition elements. It has been shown that PMMA can be electrospun into nanofiber sheets and is attractive as a sensing platform due to its nanofiber structure producing a very high total surface area for analyte interaction (Piperno et al. 2006). These nanofibers are low cost and are easy to handle so that they can be applied to our flow-through well design to test liquid or air samples. This polymer is stable over time and would be able to endure the environmental factors that will be in contact with the sensing platform. Additionally, for any future medical biosensor applications, PMMA has a high degree of biocompatibility.

Another important aspect of our sensor design is the immobilization of biomolecules to act as a potential sensing platform. We have previously used cleavable peptides that are specific to target analytes as a sensing mechanism (Law and Tung 2008). This method has been used for in-solution testing using different nanoparticles, including silica nanoparticles, quantum dots, and gold nanoparticles as sensor platforms. The ability to achieve protease interaction with the peptide constructs has been shown (Grant et al. 2007). In our present research, nanofibers are being investigated as possible

sensor platforms. The creation of a 3-D architecture is hypothesized to contribute to a higher degree of substrate loading and thus enhanced sensitivity. The utilization of nanostructured supports with immobilized biomolecules has been utilized with great success, especially for enzymatic biosensors (Wang et al. 2009). Our peptide constructs are dually labeled with fluorophores (donor and acceptor) on either side of an enzyme-cleavable region to allow for fluorescent sensing which should enhance the sensitivity of the system. The substrates are capable of using fluorescence resonance energy transfer (FRET) to detect the presence of protease by monitoring the donor and acceptor signals. These fluorescent labels on the immobilized peptides will be utilized to measure the fluorescent enhancement revealed from the nanofiber platforms. Although we are not testing the nanofiber sensor response to protease, the peptide construct was tested in solution before immobilization to the nanofibers.

Lastly we have merged the nanofibers to a sensor design that utilizes modified glass and PDMS wells to allow for flow through sensing. PDMS microchannels have been used for microfluidic assays involving electrospun nanofibers (Yang 2008). Expanding upon earlier research, we drilled a hole in a glass slide using a small drill bit (1mm) and electrospun the nanofibers onto the glass substrate. A PDMS well was then affixed over the nanofibers allowing only a small sample area to be exposed for interaction with our analyte. The advantage of using nanofibers lies in its ability to easily flow a solution or gas through its matrix allowing for analyte interaction in all three dimensions (Ren et al. 2006).

Peptide substrates were immobilized to the nanofibers and to plain glass slides to test the fluorescence enhancement from the added surface area of the nanofibers. Our hypothesis is that nanofibers acting as sensor platforms will increase the sensitivity of the sensing system by providing increased immobilization of the biorecognition molecule and allow for more interaction with potential analytes.

6.2 Materials and Methods

6.2.1 Chemicals and other materials

Poly methyl methacrylate (PMMA) (120,000) was purchased from Sigma-Aldrich (St. Louis, MO). The peptide was synthesized and purchased from 21st Century Biochemicals, Inc. (Marlboro, MA). Porcine trypsin, Isopropanol, hexamethylene diamine, borate buffer, and PBS were also from Sigma-Aldrich (St. Louis, MO). The cross-linker sulfo-GMBS was purchased from Thermo Scientific (Rockford, IL). 5-Caboxyfluorescein and 7-amino-4-methyl-coumarin-3-acetic acid were used as the fluorophores on the peptide.

6.2.2 Peptide synthesis and characterization

The peptide was designed and synthesized as trypsin substrates for future protease detection assays. The full sequence for peptide construct, CF6d, is 5CF-Ahx-CPRG[K-AMCA]-amide. The CFd6 peptide construct contained 5 amino acids and contains the (Arginine-Glycine) enzyme-sensitive region. The peptide contains fluorophores at both ends with a coumarin dye (donor) at its n-terminus and a fluorescein dye (acceptor) at its c-terminus. The cleavable region is specific to the protease and will cause the substrate

to separate between the arginine and glycine, releasing the portion of the peptide that contains fluorescein from the immobilized portion of the peptide in the presence of trypsin.

Initially the peptides that will be immobilized to the nanofibers were analyzed in solution to determine cleavability and response. The results gave us a benchmark to compare in-solution activity with the response when immobilized to a solid substrate. An 85 pM amount of CF6d peptide in PBS was tested with different doses of trypsin (1, 5, 10, 20, 50, 100, 500 and 1000 nM). Cleavage of the peptide results in a FRET change between the donor and acceptor signal. This ratio can be analyzed to determine the response of the sensor and a dosage response curve was generated.

6.2.3 Preparation of PMMA solution

We investigated different protocols to prepare PMMA solution for electrospinning including using different concentrations, solvent mixtures, and utilizing heat treatments (Manesh et al. 2008; Piperno et al. 2006; Wang et al. 2002). The material concentration and molecular weight greatly modifies the nanofiber diameter and uniformity. We experimented with concentrations from 0.5 – 1.5 grams in 10 ml of solvent, different solvents and ratios of solvents (acetone and DMF), and different temperatures (room temperature and 60°C) for the electrospun solution. Nanofibers with concentrations below 1.0 grams PMMA were very thin but discontinuous and weak. Above 1.0 grams lead to nanofibers with larger diameters and a difficulty in the ability to spin the solution. The protocol that was used in this research to prepare the PMMA

solution for electrospinning is as follows: One gram PMMA was added to a small glass vial containing 10 ml acetone. We sonicated the solution for at least 2 hours until the solution was homogenous. The solution was allowed to cool to room temperature before spinning. The rheological properties of the PMMA solution was evaluated using on a BrookField DV+II Viscometer at 25°C prior to electrospinning and are shown in Figure 6.2.

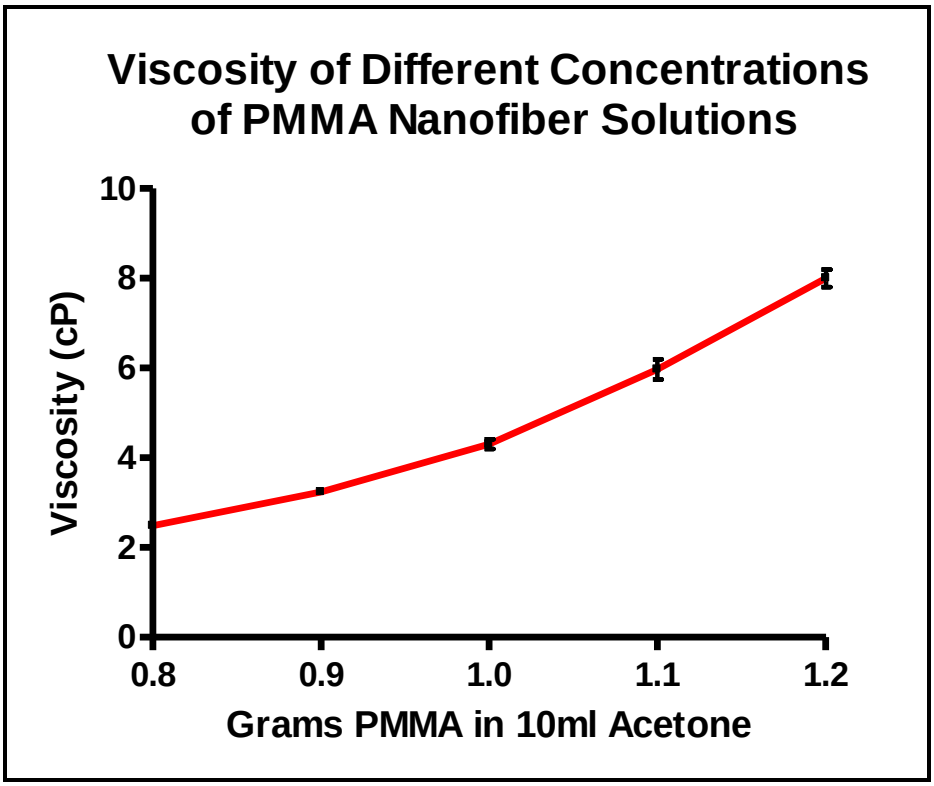

Figure 6.2 – Viscosities of Different PMMA Solution Concentrations.

6.2.4 Electrospinning of PMMA nanofibers

Electrospinning is a fairly easy method to achieve nanosized fibers. Although the process is elemental, there are many parameters which need to be investigated to produce continuous, uniform nanofibers. Some of these parameters include the deposition time, flow rate, applied voltage, and separation distance between the needle and the collection plate. Other factors like the needle size, type of collection method or plate size/material/location, and the internal atmosphere (air temperature, humidity, air composition) could also be addressed to modify the fibers. Deposition time varies between a few seconds to minutes depending upon the required thickness layer (Frenot and Chronakis 2003). We have found that a very light layer forms (1-2 μm) after about 30 seconds, 1 minute for a medium thickness layer (3-5 μm), and 2 minutes or more for a thicker layer (5 μm or more). Figure 6.3 shows electrospun nanofibers on full glass slides electrospun for different lengths of time.

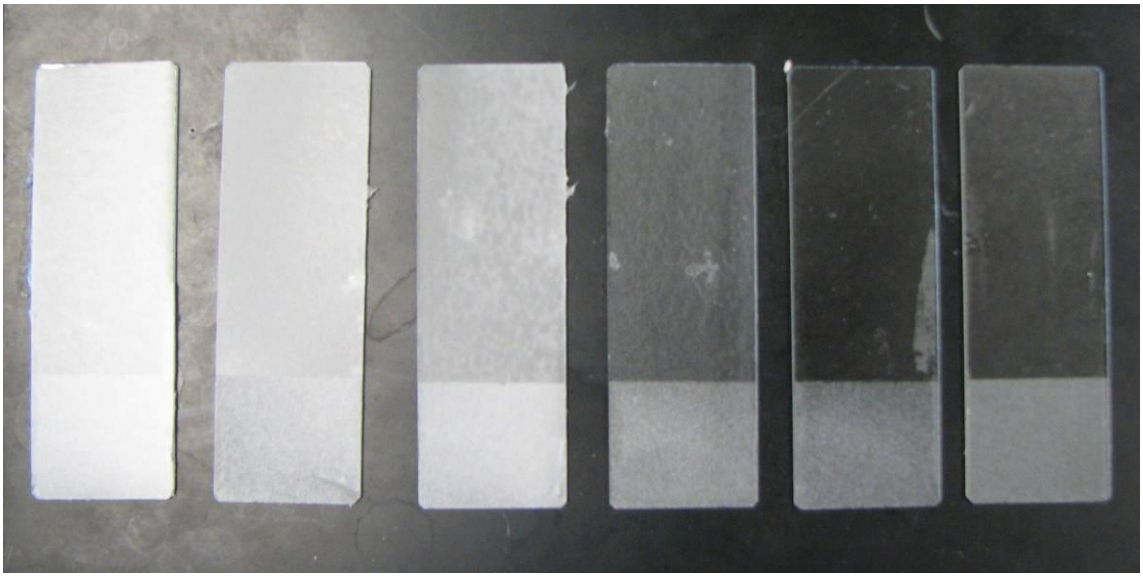

Figure 6.3 – PMMA Nanofibers electrospun for different lengths of time starting at 5minutes, 3mintues, 2minutes, 1minute, 30seconds, and 10seconds.

Reproducibility can be a problem with the PMMA nanofibers due to the buildup of solution on the tip of the needle. This can be avoided via employment of an acetone bath in order to minimize the effect of the solution drying on the tip of the needle, which impedes the deposition on to the collection site. Flow rates were investigated between 1-5 ml/hr and we have found that 5 ml/hr is the optimized rate to achieve uniform and continuous nanofibers. Lower flow rates caused discontinuous nanofibers and higher flow rates only caused solution build-up on the needle and slightly non-uniform fibers. An applied voltage between 15-25 kV has been used and it was found that the ideal voltage is around 20 kV. This voltage is the critical voltage that begins to pull the polymer solution into nanofibers. The separation distance used was 18 cm, but we tested distances from 10-20 cm and observed only small differences in nanofiber structure. We used an 18 gauge disposable metal needle and a copper plate as our collection platform. The fibers were collected as sheets on glass slides and we have also fashioned other unique collection plates like modified glass with PDMS wells. Certain nanofibers require additional processing after they are spun including drying and/or functionalization procedures. The PMMA nanofibers were dried overnight at 70°C, which also aids in making them more transparent. The nanofiber samples were characterized using light microscopy and SEM (Scanning Electron Microscopy). The nanofibers diameter was measured using a SEM micrograph and averaging the diameters of 10 nanofibers. The specific surface area was found using a Brunauer-Emmett Teller (BET) nitrogen absorption test.

6.2.5 Functionalization of PMMA nanofibers

The PMMA nanofibers contain methyl groups on its surface and needed to be functionalized in order to immobilize peptides to their surface. We utilized a method proposed by (Fixe et al. 2004) using hexamethylene diamine to create amine groups from the methyl groups. This group chemically modified bulk films to create aminated-PMMA. The nanofibers were first cleaned in a solution 95% Isopropanol for a few minutes before being rinsed with DDH_2O. The nanofibers were then soaked in a 10% solution of hexamethylene diamine in 100 mM borate buffer (pH 11.5) for 2 hours. The samples were washed in borate buffer (pH 11.5) for 15 minutes and then in borate buffer (pH 8.0) for 15 more minutes. These samples were allowed to dry overnight at 30°C and then FTIR was used to access the functionalization protocol.

6.2.6 Immobilization of peptides to PMMA nanofibers

The peptide substrates were immobilized to the PMMA nanofiber surface on glass slides using a chemical binding method involving 1 mM sulfo-GMBS. This links the aminated-PMMA to the sulfhydryl on the cysteine of the peptide. A 20 µM amount of CF6d peptide was determined to be the optimized concentration in order to achieve an adequate signal from the optical detection equipment but within a range that leads to the lowest limit of detection. Excessive immobilization of the nanofiber surface can cause steric hindrance and can lead to peptides that may leach out contributing to strong background signals. Using a lower concentration of peptide substrates would contribute to a lower limit of detection due to the ability of lower concentrations of analyte eliciting a response. Fluorescence characterization to monitor the increase in peptide

immobilization was performed using a fluorescent microscope. We compared fluorescent data between the nanofiber platforms and the planar glass surfaces.

6.2.7 Design of the flow-through sensor

We have designed a flow-through sensor based on PDMS wells used in conjunction with modified glass and nanofibers. Nanofibers were cut and placed on glass slides with a small hole drilled in them (approximately 1mm) and then covered with a PDMS well with dimensions of 400 µm x 400 µm. This allows a small opening for liquid or gas samples to flow through the fiber mesh to interact with the immobilized peptide substrates. Functionalization and immobilization was also carried out on the flow-through sensor design and testing was performed using a fluorescence microscope using an integration time of 500ms.

6.3 Results

6.3.1 Characterization of peptides in solution

The peptide constructs that were tested had 5 amino acids. These short peptides offer the advantage of placing the fluorophores in closer proximity for more efficient energy transfer as well as lowering cost and preventing folding of the peptide substrate, which would hinder the cleavage site. The shorter peptide has not lead to any adverse effects from the analyte not being able to access the cleavage site. The peptides were initially tested in-solution with different concentrations of trypsin and the results are shown in Figure 6.4. The measure of the donor intensity over the acceptor intensity was calculated and the percent change between the initial value and the final value. A dosage

response curve was generated and the limit of detection was found to be 20 nM trypsin, which is the concentration that shows a significant response over the baseline.

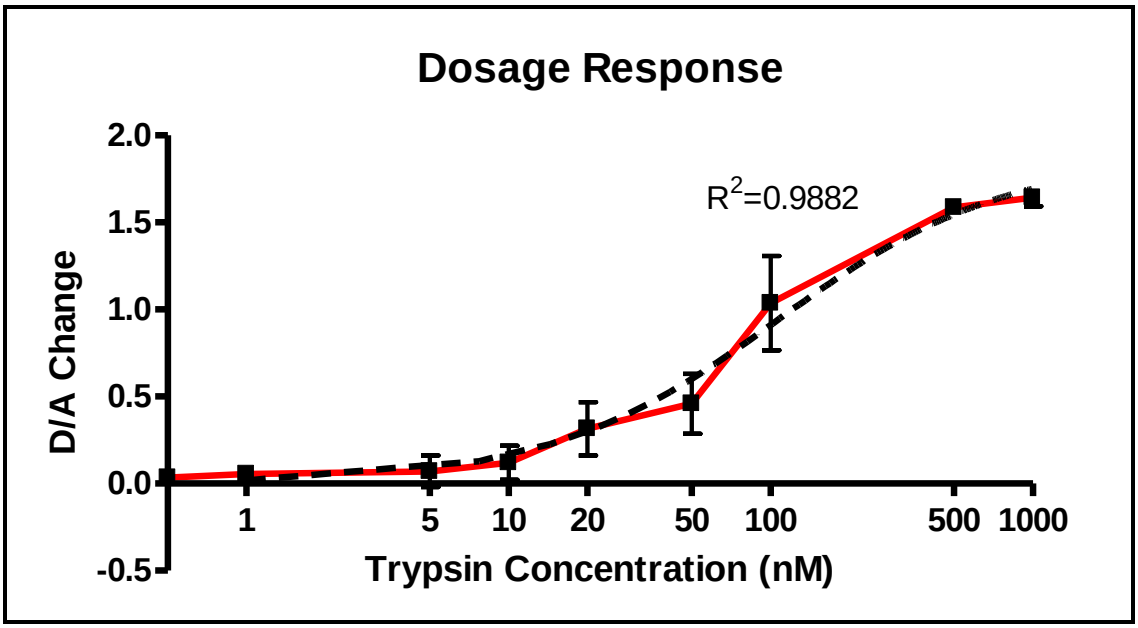

Figure 6.4 – Dosage Response of CF6d Peptide in Solution.

6.3.2 Characterization of PMMA Nanofibers

The PMMA nanofibers were characterized initially with a light microscope to determine uniformity and structure of the nanofibers produced. These images assisted in setting up the solution and spinning parameters. We determined that a low concentration of PMMA resulted in extremely thin fibers around 300 nm, but that they were discontinuous and non-uniform. Higher concentrations of PMMA resulted in uniform fibers but with larger than desired diameters above 2 µm. We found that using 1.0 grams of PMMA in 10 ml acetone produced the best nanofibers for thin, consistent sensing platforms using our electrospinning parameters. These parameters were determined to be 20 kV applied voltage, 5 ml/hr flow rate, 18 gauge needle, and 18 cm separation distance.

The surface chemistry of the nanofiber samples was verified using FTIR before and after the functionalization step to determine the formation of the amine group on the PMMA nanofibers. A characteristic amine peak was present at 1580 cm^{-1} in the scan after the protocol was completed on the PMMA nanofibers indicating a successful functionalization shown in Figure 6.5. The size and structure of the nanofibers were confirmed using SEM micrographs. These results can be found in Figure 6.6. The fiber sizes ranged from 500 nm to 2 μm with the average fiber diameter being about 1 μm based on 10 measurements. Lastly, the results of the BET nitrogen absorption experiment found that the nanofibers possessed a specific surface area of 12 m^2g^{-1}. Although the specific surface area is not as large as nanoporous films many sensing mechanism (peptide cleavage) cannot properly function within nano-sized pores (Wang et al. 2009).

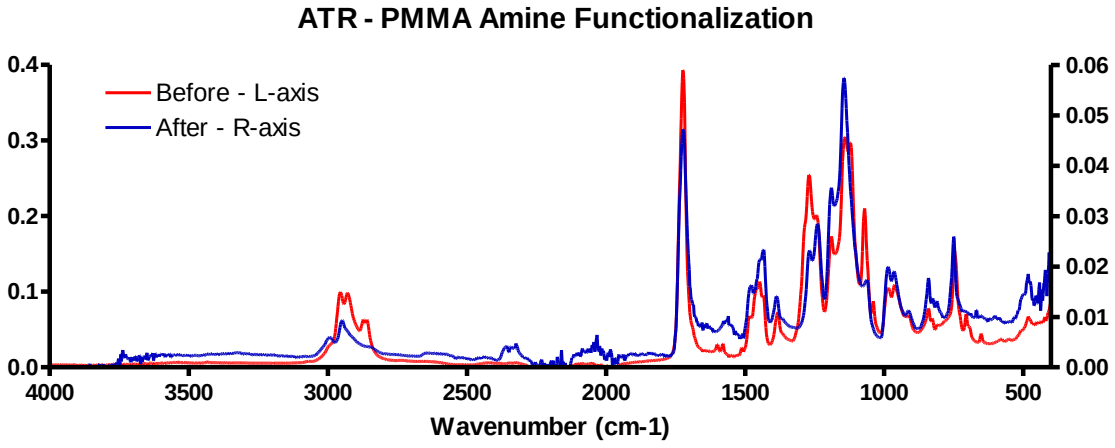

Figure 6.5 – ATR of PMMA before and after Functionalization Protocol.

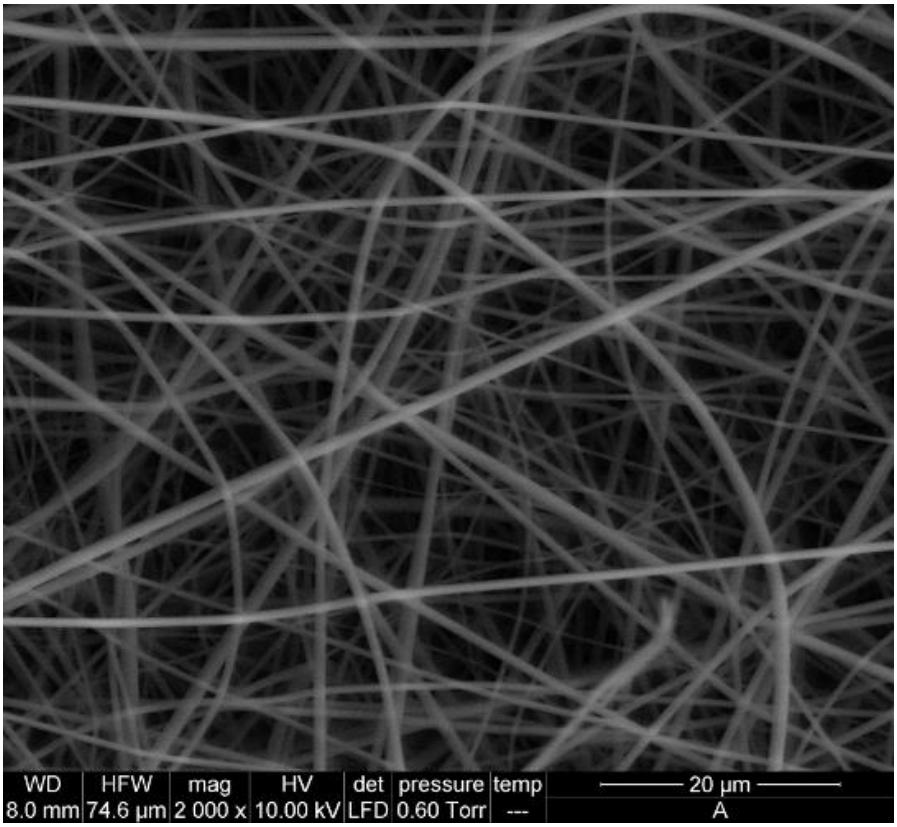

Figure 6.6 – SEM Micrograph of PMMA Nanofibers.

6.3.3 Characterization of peptides immobilized to PMMA nanofibers

A fluorescence microscope (500ms) was used to analyze the peptide immobilized PMMA nanofibers. Initial experiments were performed on the nanofibers to determine the optimal amount of peptides to immobilize to the nanofibers and to determine if leaching occurs. The increased surface area of the nanofibers allowed for more peptide to immobilize by volume with a 170% increase in signal over continuous thin-film platforms. A fluorescence micrograph and the SPEX data are shown below in Figure 6.6.

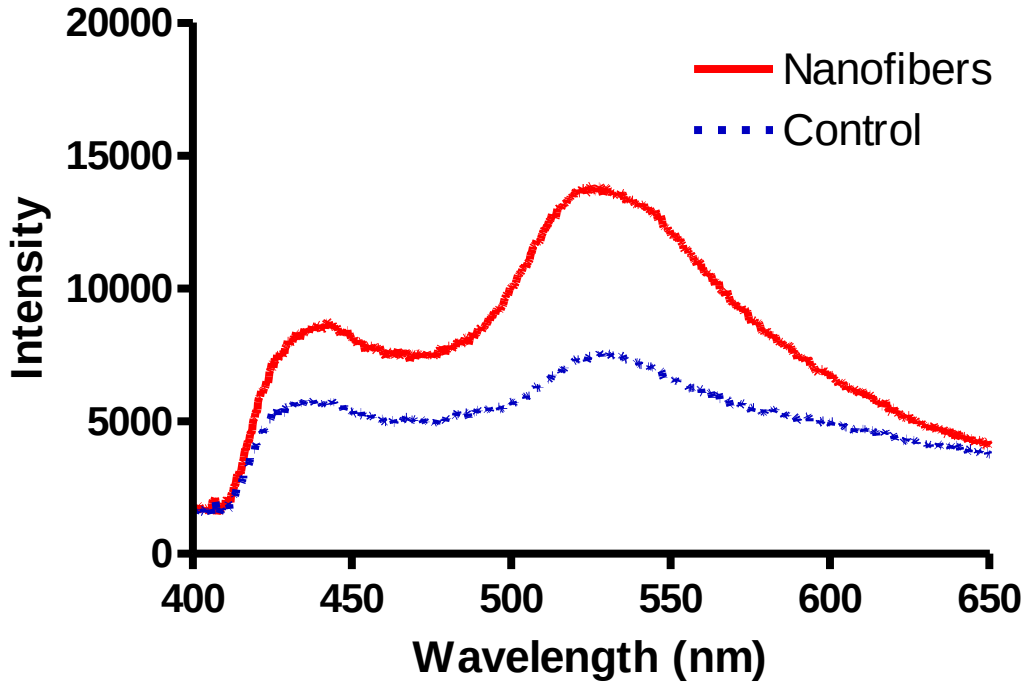

Figure 6.7 - Immobilized Nanofibers vs. Glass Slide Control.

6.4 Discussion

PMMA nanofibers have been designed to be utilized as a sensing platform for peptide-based biosensing. They have the distinctive property of exhibiting higher surface area than planar films due to their multiple layers increasing the surface area and giving rise to porosity; thus, making them ideally suited for sensitive detection system (Manesh et al. 2008). Due to the nanosized fibers, we achieved an increased surface area for immobilization of the peptide and 3-dimensional interaction between the analyte and peptide substrate for the potential of significantly increased sensitivity levels of the biosensor. Nanofibers seem well suited due to the ability to provide a fibrous mesh creating pores for liquids and aerosols to permeate through the network (Ren et al. 2006).

Electrospinning has proven to be a sufficient method to acquire nanofibers easily and cheaply. The nanofibers are strong enough to be handled and can be modified to provide attachment sites for sensing substrates. Individual parameters must be set for each polymer solution that is electrospun and operating factors must be determined to spin nanofibers with the most favorable properties. The solution viscosity related to the concentration of PMMA in the solution can have a grave impact on the final diameter of the fibers. Also certain spinning parameters affect the final morphology of the nanofiber mesh. We prepared many sample solutions and worked with each of the spinning parameters to determine the appropriate procedure to electrospin nanofibers with the most conducive properties for sensing applications. Our nanofiber platforms were uniform and had fiber diameters ranging from 500 nm to 2 μm. This allows for the sensing surface-to-volume ratio to be larger giving the ability for more peptide substrates to bind in a sample volume, while also permitting solutions and gases to freely flow through the matrix. However, problems with porous surfaces due to molecules being trapped in the inner pores have resulted in further research to investigate these nanofiber matrix (Wang et al. 2009).

Our sensing mechanism used a fluorescence change from a FRET labeled peptide construct that was designed for a particular analyte. Peptides were easily immobilized on the surface of the nanofibers after following functionalization and cross-linking protocols. The appropriate concentrations of peptide to immobilize on the nanofibers were determined. An excess amount of peptide may contribute to the background signal due to weakly bound substrates leaching over time. Steric hindrance might also become a

problem if too many peptides are immobilized to the nanofiber surface. The nanofibers were immobilized with 20 µM CF6d peptide and thoroughly washed to prevent any loosely bound peptides from leaching during signal transduction. We were able to enhance the fluorescence signal greatly from the labeled peptide substrate on nanofibers over planar thin films with an intensity increase over 170%.

6.5 Conclusion

With the advent of optical biosensing and the emergence of nanotechnology, a novel method of electrospinning nanofibers as a platform for peptide-based biosensors capable of flow-through detection of analytes has emerged. Our design of a flow-through biosensor based on utilizing PMMA nanofibers and peptide substrates has proven to be an acceptable platform technology. We have found the optimized properties of suitable nanofiber scaffolds and have determined the proper immobilization protocol for peptide attachment. An increase in fluorescence intensity of 170% from the nanofiber platforms over thin film samples is proof that we have a potential very sensitive sensing platform. With further research this sensing platform and sensing mechanism could provide a viable method for protease detection.

Acknowledgements

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Chapter 7

TRYPSIN DETECTION UTILIZING PEPTIDE SUBSTRATES IMMOBILIZED ON PMMA NANOFIBERS

Abstract

We have brought together a unique sensing mechanism and sensing surface for the development of a flow-through sensor for protease detection. Cleavable peptides constructs were designed because of their enhanced performance over antibody systems to detect various analytes. Synthetic peptides are smaller and are more stable in non-aqueous environments than antibodies and can be designed to be very specific to target analytes. The peptide constructs are immobilized to electrospun PMMA (polymethyl methacrylate) nanofibers, which possess large surface area and allow for 3-dimensional interaction with the analyte. PMMA is chemically modified leaving a functionalized surface which is then crosslinked to the terminal end of the peptide constructs. The use of a dual-labeled peptide constructs provides the ability to detect protease analytes by monitoring changes in the fluorescence signal upon cleavage of the constructs by the protease. We have built a sensing system using glass slides and PDMS (polydimethylsiloxane) wells that allow for liquid or gas samples to flow through the sensing system. The sensor system was tested using fluorescence microscopy and a built optical detection system has been constructed for remote deployment using the nanofiber sensor chips. We have successfully detected trypsin down to 100 pM with response time within 5 minutes. Additional peptide substrates can be designed for detection of many

other various analytes and potentially could help resolve problems with detection equipment used in biothreat detection and medical applications.

7.1 Introduction

Nanofibers have been found to be ideal sensing platforms due to their relative size and structure allowing for a high surface-to-volume ratio and 3-dimensional interaction of the analyte with the sensing surface (Manesh et al. 2008; Ren et al. 2006). These nanostructured designs can increase the surface area several magnitudes over planar sensing surfaces (Wang et al. 2002). Because of these advantages, biosensors utilizing nanoplatform designs have been popular for at least the last decade. Nanoparticles, nanofilms, and nanofibers have all been employed as nanoplatforms for biosensor applications.

Nanoparticles have been utilized mostly for in-solution testing, where the molecular recognition element would be immobilized to the nanoparticles and tested in solution. This led the way for nanomaterials to continually break barriers in sensitivity with detection limits reaching the femto-level (Mahmoud et al. 2008; Tripp et al. 2008; Wang et al. 2008). However, in-solution testing is not always practical for some applications, and thus films based on nanoporous substrates have been developed. The nanosurfaces are designed with very small pores (<10nm) to significantly increase the surface area and allow for lower limits of detection (Rossi et al. 2007; Thoelen et al. 2008). Some problems with these nanofilms are that they have a tendency to crack, and there is a limit to how small the pores can be to allow for interaction of the analyte with the surface. If the analyte cannot penetrate the pore then it cannot come into contact with the biomolecule that is eliciting a response. The sensing mechanism becomes buried in the pore and steric hindrance prevents the sensor from functioning properly.

Nanofibers overcome some of these issues by increasing the surface area via the nanosize diameters of the fibers, which allow high surface to volume ratios. Additionally, the multiple layers of fibers contain large pores for gases and liquids to flow through the matrix while the rod-like structure is exposed on all sides to allow for interaction with the analyte. The surface area increases with the increasing depth of nanofiber layers resulting in the potential of detecting low limits of detection. The polymer nanofibers are processed to reduce cracking; although mild cracking of a few nanofibers will not cause the entire platform to become unstable. Examples of sensors that utilize nanofiber platforms include the detection of explosives, urea, and glucose to name a few (Manesh et al. 2008; Ren et al. 2006; Sawicka et al. 2005; Wang et al. 2002; Yang 2008). Quenching-based optical chemical sensors have been fabricated with orders of magnitude higher sensitivity to 2,4-dinitro toluene (DNT) than from continuous thin films. Advancements in response time and lower sensitivity were discovered using electrospun biocomposite nanofibers for urea biosensing. Multiple groups have worked on glucose detection using electrospun membrane platforms. Lastly microfluidic immunoassays for HIV based on electrospun nanofibrous membranes were used to improve sensitivity and signal-to-noise ratio. Nanofiber platforms are beginning to significantly impact in the biosensor industry due to their benefits over planar sensing surfaces.

We are employing synthetic peptides as protease substrates for a fluorescence sensing method which utilizes a FRET (Fluorescence Resonance Energy Transfer) technique to detect potential analytes. The method has been used previously by our

group as well as others (Grant et al. 2007; Sapsford et al. 2008a). Peptides were designed as trypsin substrates and were dually-labeled with a fluorophore on each of their terminus in close proximity so that when the peptide is initially intact an energy transfer can occur between the fluorophores (Grahn et al. 1998). The fluorophore attached to the peptide at the binding site to the nanofiber is a coumarin dye and is termed the donor, while the fluorophore on the opposite terminus of the peptide is a fluorescein dye and is termed the acceptor. Exciting the donor molecule in close proximity to the acceptor will cause energy transfer to occur resulting in a predominantly acceptor signal emission. Proteases interact by cleaving a certain amino acid sequence, between arginine and glycine for trypsin, causing the peptide substrate to separate. Detection can be determined by a change in fluorescence signal indicated by the decreased acceptor signal and the recovery of the donor signal emission. The ratio of these peaks can be analyzed to determine the presence of analyte to very low concentrations. We utilized peptides because they are smaller and are more stable in non-aqueous environments and because of the potential use as a remote deployable sensing system (Soper et al. 2006) for possible chemical and/or biological detection.

The previous chapter studied the optimized nanofiber structure to maximize the signal for peptide immobilization. The nanofiber sensing platform was designed with small diameter fibers to maximize surface area and to allow for liquids and gases to flow through the matrix and allow interaction in 3-dimensions rather than in 2-dimensions from most planar substrates. A schematic for the sensor system is shown in Figure 7.1 and the top view is shown in Figure 7.2. A solution can be passed through the openings

between the nanofibers and the analyte is allowed to interact with the nanofiber surface containing the peptide substrates. A positive response occurs when a protease cuts the peptide substrate in two and the terminal portion floats away causing a change in fluorescence observed due to the terminal acceptor fluorophore being separated in distance from donor fluorophore. The fluorophores when not in close proximity cannot allow energy to transfer between the molecules resulting in a decrease in acceptor emission and an increase in donor emission.

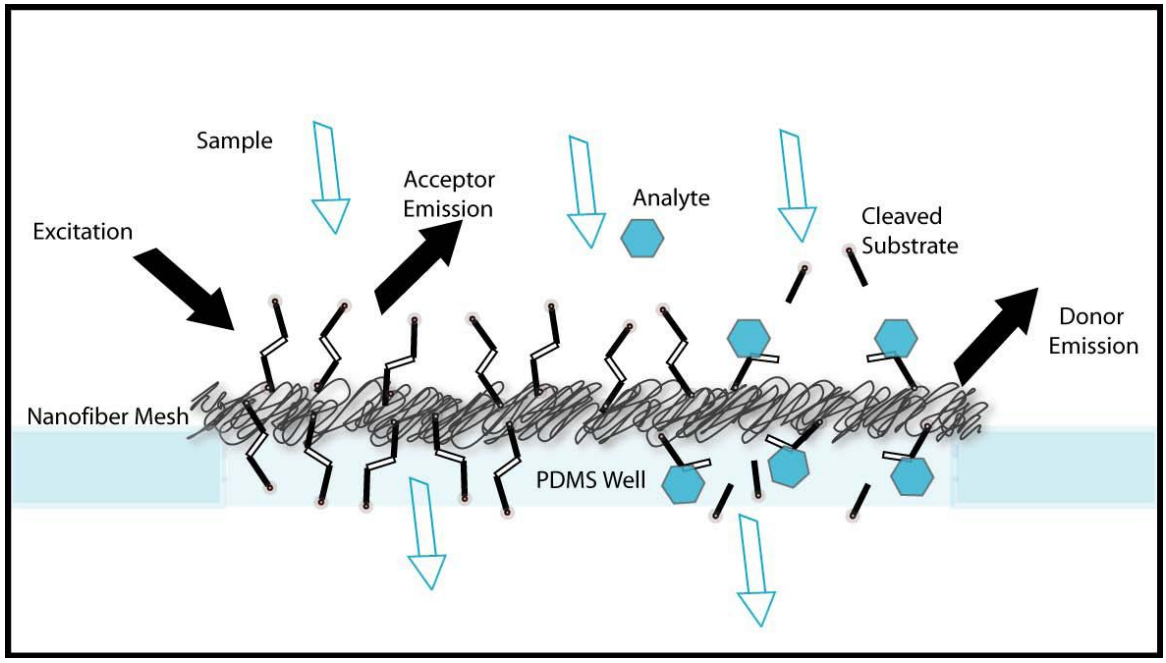

Figure 7.1 – Schematic of Nanofiber Platform Biosensor.

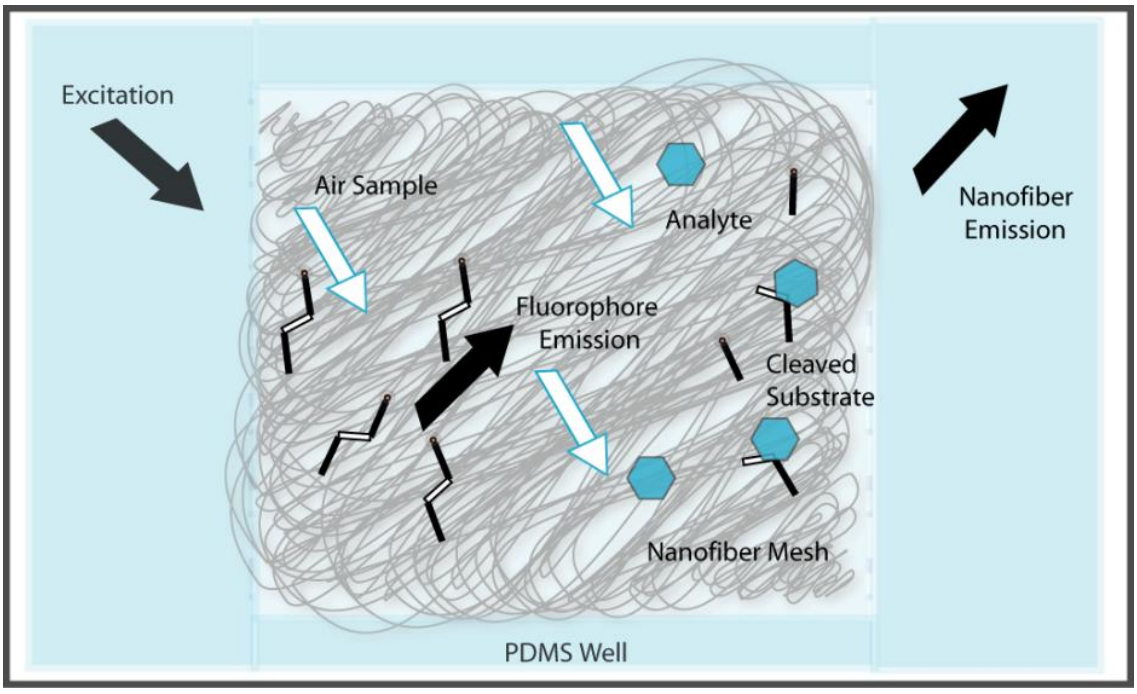

Figure 7.2 – Top View of Nanofiber Sensing Platform

We have thoroughly tested the nanofibers and have modified their surface to attach the peptide substrates. This chapter will show the results of a test of a trypsin substrate immobilized on PMMA nanofibers. We have tested the sensing system in order to optimize the sensitivity of the sensor. The limit of detection has been found to be down to 100 pM trypsin and detection within a few minutes. This sensor design has the potential to detect a vast amount of potential analytes including bio-threat agents and is designed to be remotely deployed to protect against bio-terrorism.

7.2 Materials and Methods

7.2.1 Chemicals and other materials

Poly methyl methacrylate (PMMA) (120,000), porcine trypsin, isopropanol, hexamethylene diamine, borate buffer, and PBS were purchased from Sigma-Aldrich (St.

Louis, MO). The peptides were synthesized and purchased from 21st Century Biochemicals, Inc. (Marlboro, MA). The cross-linker GMBS was purchased from Thermo Scientific (Rockford, IL). 5-Caboxyfluorescein (Em 520nm) and 7-amino-4-methyl-coumarin-3-acetic acid (Em 450) were used as the fluorophores for the FRET pair.

7.2.2 Peptide synthesis

We designed and purchased the peptide as a trypsin substrate in the design of the protease biosensor from 21st Century Biochemicals (Marlboro, MA). The full sequence for CF6d is 5CF-Ahx-CPRG[K-AMCA]-amide. The CFd6 peptide construct contained 5 amino acids and contains the (Arginine-Glycine) enzyme-sensitive region where trypsin will cut the sequence. The peptide contains fluorophores at both ends with a coumarin dye at its n-terminus and a fluorescein dye at its c-terminus. The substrate contains a cleavable region that is specific to the protease and will cause the substrate to separate between the arginine and glycine, releasing the portion of the peptide that contains fluorescein from the immobilized portion of the peptide in the presence of trypsin. The peptide constructs were initially tested in solution to determine cleavability and limits of detection with results shown in a previous chapter demonstrating 20 nM LOD.

7.2.3 Preparation of PMMA Nanofibers

Electrospinning is a technique to acquire nanofibers by electrically charging a polymer solution and pulling it to a collection plate. PMMA was the polymer that was selected due to its optical properties and due to its ability to be chemically modified. We

investigated many methods of electrospinning PMMA and developed our protocol from these papers and from testing results (Frenot and Chronakis 2003; Piperno et al. 2006). Briefly we prepared the polymer solution using 1.0 grams PMMA in 10 ml acetone. This solution was sonicated until it was homogenous. The cooled solution was then electrospun at an applied voltage of 20 kV with a flow rate of 5 ml/hr for 1 minute collected on glass slides (15mm x 25mm). An acetone bath was placed in the electrospinning chamber to reduce build up of the polymer drying on the tip of the needle. The electrospun PMMA nanofibers were then heat treated at 70°C overnight. These fibers were then chemically modified to create an aminated surface using 10% hexamethylene diamine using a protocol from (Fixe et al. 2004). The amine functional group was then linked to using the GMBS crosslinker (1mM) which in turn was then immobilized to the peptide (20µM). The PMMA nanofibers were fully characterized optimizing its structure for sensing applications by investigating solution parameters and electrospinning technique to produce very thin, uniform fibers capable of enhanced signal. The functionalization protocol was also characterized to assess the chemically modified surface for peptide immobilization. The nanofiber platforms were electrospun, processed, functionalized, cross-linked, and then immobilized with peptides to determine response and limits of detection for protease sensing. The PMMA nanofibers with immobilized peptides were characterized with a fluorescence microscope (500ms integration time) to measure its fluorescence intensity.

A nanofiber sensor design was developed with glass slides and PDMS (polydimethylsiloxane) wells that allow for liquid or gas samples to flow through the

sensing system. This design is based off previous designs using PDMS for microfluidic flow through microchannels (Yang 2008). The process to build the sensor is shown in Figure 7.3 and the built sensor is shown in Figure 7.4. We also tested the sensor using a fluorescence microscope with UV excitation using 500ms integration times.

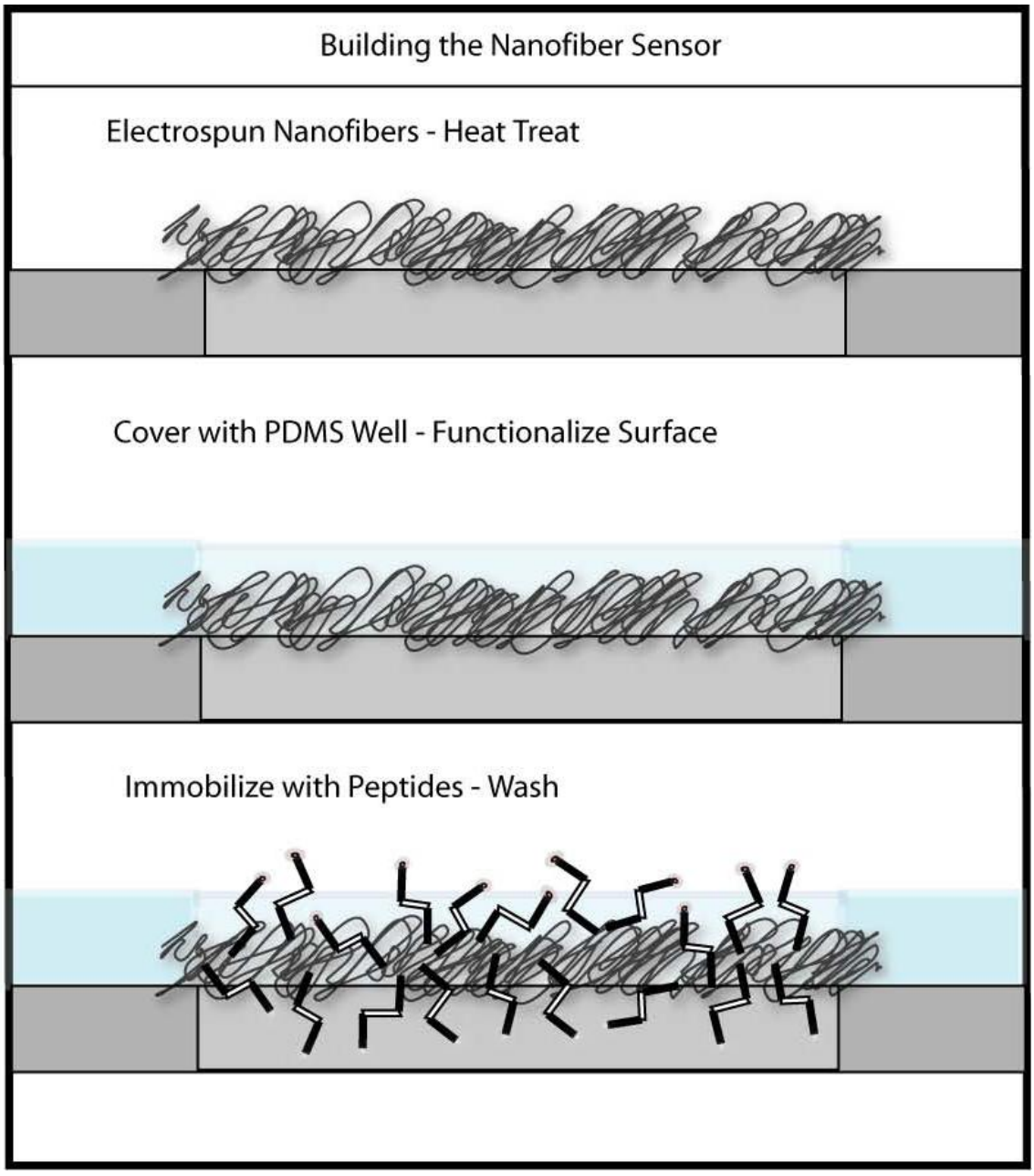

Figure 7.3 – Building the Nanofiber Sensor.

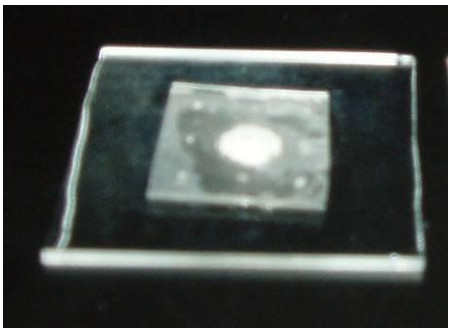

Figure 7.4 – Built Flow-through Nanofiber Sensor.

7.2.4 Response of peptide constructs to enzymes

The nanofibers with immobilized CF6d peptides were initially scanned using a fluorescence microscope with an Ocean Optics Spectrometer. The samples were then exposed to 100 pM trypsin for 5 minutes and then washed before they were scanned again for sensor response trials. The fluorescence microscope was utilized to acquire the fluorescence intensity using a UV excitation wavelength and measuring the fluorescence signal at 450nm and 520 nm. Six trials for each sample were performed.

Dosage response experiments were also investigated where known concentrations (between 10 pM – 10 nM) of the enzymes were exposed to the samples and a scans was taken after 5 minutes for each. This data was then analyzed to present a concentration dependent change in fluorescence. Changes in dosage and time response curves were produced and standardized to detect the presence of the enzymes in the samples.

7.2.5 Remote deployable optical detection system

An optical detection system that can be remotely deployed has been developed. It houses a UV-LED, a series of band filters and dichroic lenses, and detectors to excite and

collect the fluorescence used for the signal transduction. These on-hand components are somewhat low grade components and higher quality filters should be purchased to truly test this device. This lab-built set-up is being optimized for the nanofiber sensing platform, but could also be used for other planar sensing surfaces. Additionally the system was designed so that an air sampler or microfluidic tubing could be added to the detection system for automated sampling of liquids and gases. A schematic and picture of the device is show in Figure 7.5 and Figure 7.6.

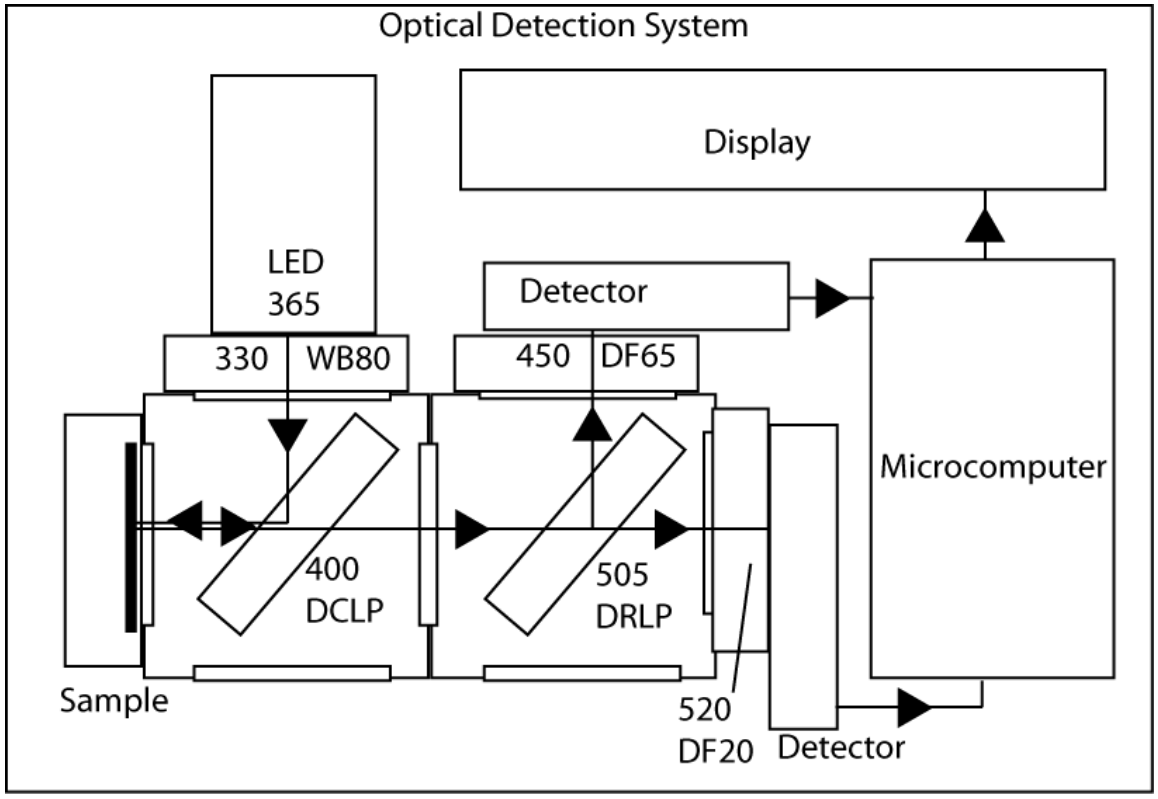

Figure 7.5 - Schematic of Optical Detection System.

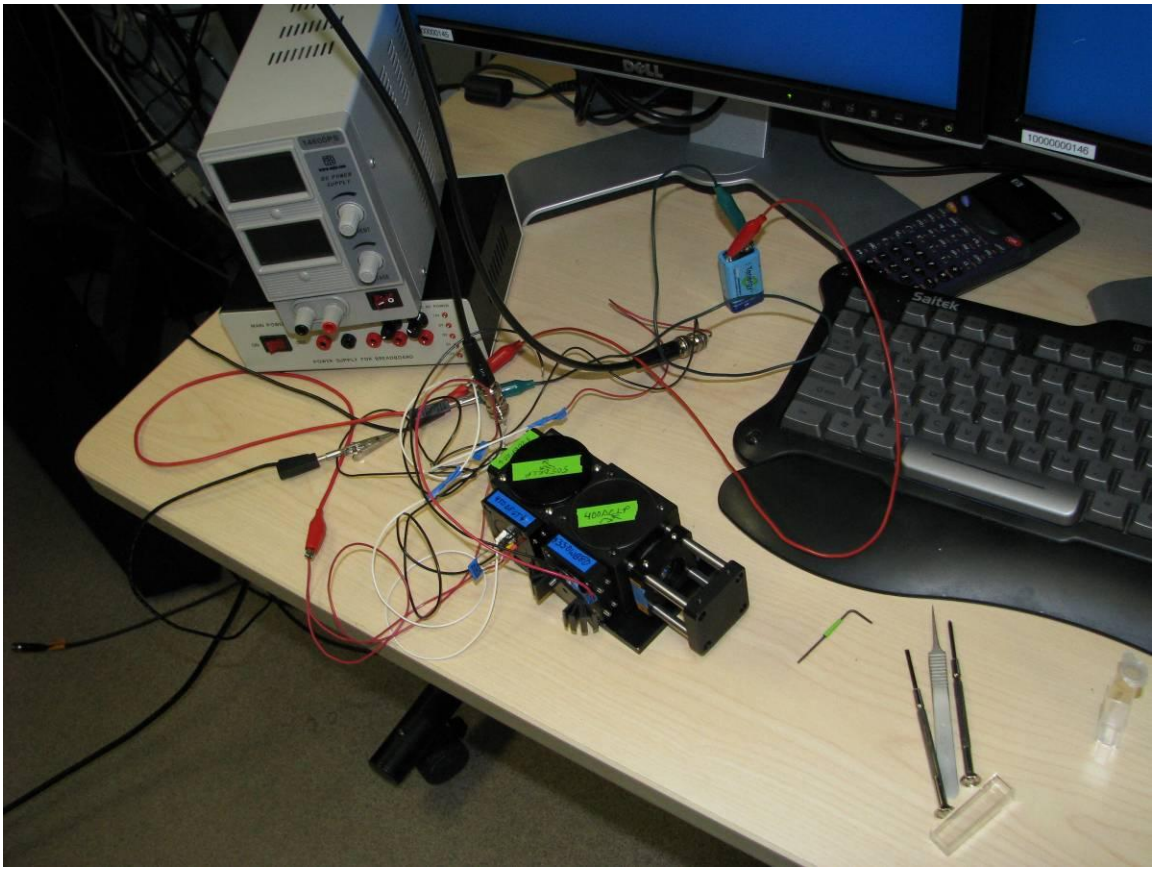

Figure 7.6 – Pictures of the Optical Detection System.

7.3 Results

7.3.1 Characterization of PMMA nanofibers and peptide immobilization

The previous chapter demonstrates the characterization of the nanofiber setup and optimization and thus will not be presented here. Nanofiber platforms were designed to have a uniform structure and very thin fibers to enhance the surface area. Nanofibers ranged from 500nm – 2 μm with an average diameter of 1 μm. FTIR was used to verify that amine groups were created on the nanofiber surface and fluorescence microscopy was used to measure the fluorescence signal intensity after the peptides were immobilized.

7.3.2 Sensor response to trypsin

The raw data of the sensor response is demonstrated in Figure 7.7 and shows the before and after for exposure to 100 pM trypsin. After trypsin is introduced the donor peak increases slightly and the acceptor peak decreases due to cleavage of the peptide substrate. The portion of the peptide containing the terminal fluorophore was washed away from the sensing surface leaving only the donor fluorophore. We analyzed different doses of trypsin (10 pM, 100 pM, 1 nM, and 10 nM) to find the limit of detection of the sensing system. We utilize the change in the fluorescence intensity of the donor/acceptor peak to create a concentration dependant curve.

Dosage response studies show that as the enzyme acts on the peptide immobilized to the nanofiber the acceptor intensity decreases while the donor signal increases. This continues over time and occurs at a faster rate with an increased amount of analtye. The dosage response study indicated a limit of detection of 100 pM trypsin with a response time around 5 minutes.

The optical detection system designed to be a portable unit was unable to detect a useable signal due to the low grade filters and because the samples were optimized for the fluorescence microscope experiments.

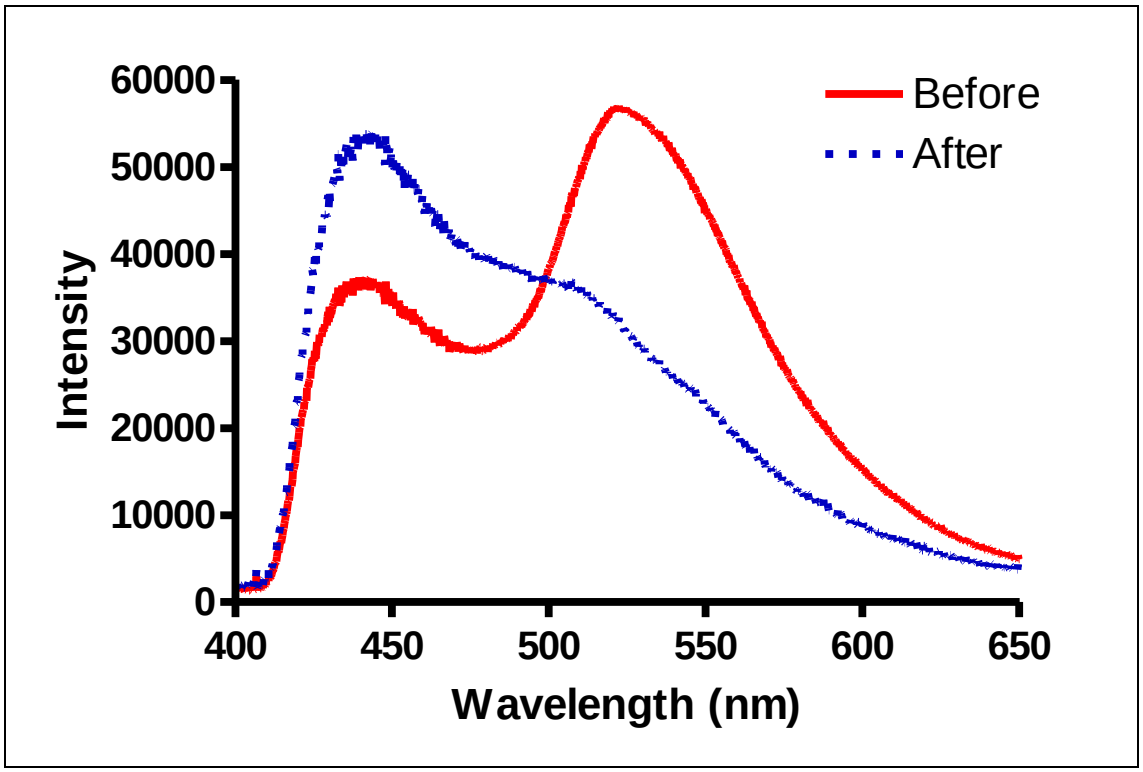

Figure 7.7 - Nanofiber Sensor Response to Trypsin.

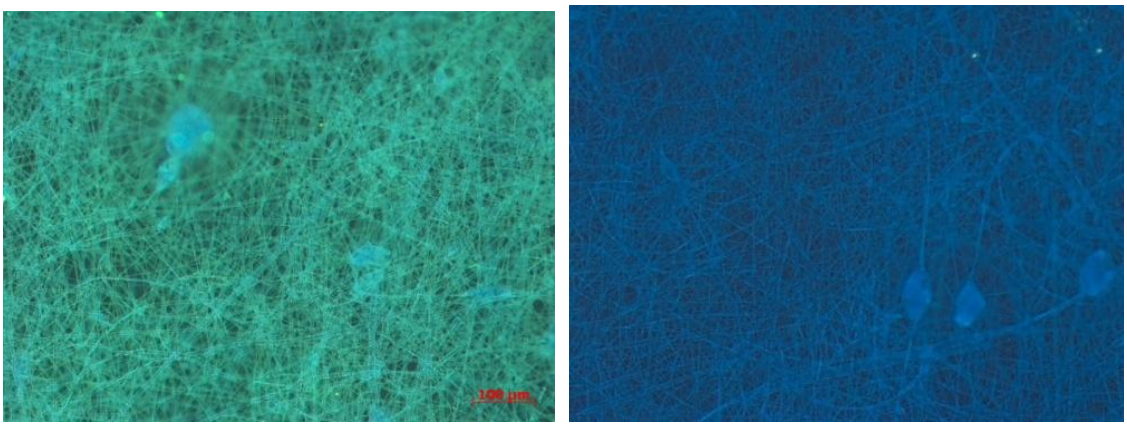

Figure 7.8 – Fluorescent Microscope Images of Nanofibers before and after Trypsin.

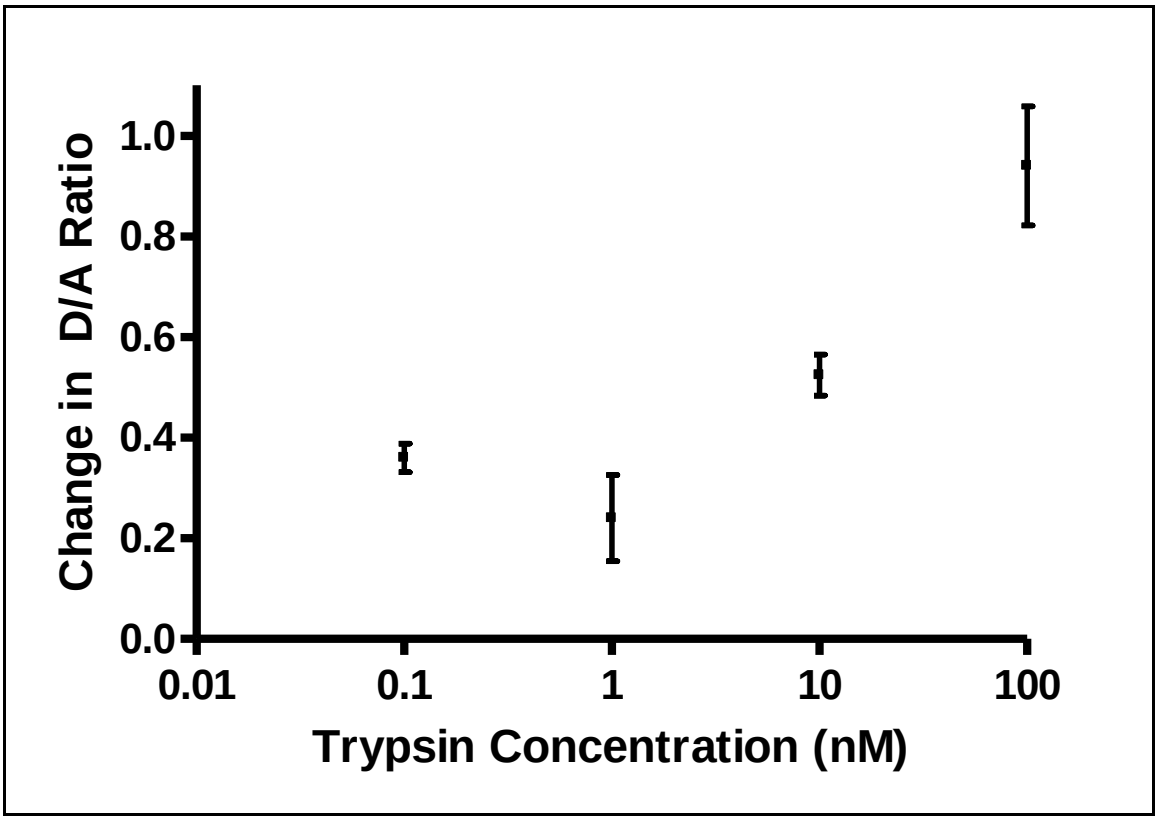

Figure 7.9 - Dosage Response of Nanofiber Sensor to Trypsin.

Unless otherwise noted, all statistical analysis will be carried out using GraphPad Prism version 4.0. A two-way ANOVA was performed for a comparison test between the positive and control substrates with significance set at $p<0.05$. We found that there is a significant change between the CF6d (positive) substrate and control substrate with addition of trypsin. The control (not shown on the graph) was no trypsin added to the nanofibers and its change in D/A ratio value was 0.058667±0.02223.

7.4 Discussion

Utilizing the nanofiber platforms for trypsin detection has significantly lowered the limit of detection by 2-orders of magnitude and has reached the picomolar range from

our previous in-solution results. Nanofibers have been found to increase the surface-to-volume ratio allowing for more peptides to immobilize to its surface and the 3-dimensional matrix allows for increased interaction of analyte with the peptide substrate. The flow through design was tested with liquid samples, although air samples should interact in a similar manner. Depending on the analyte and how it functions air samples might also need to be impinged into a liquid or an air sample might need to be preconcentrated to illicit a response.

Nanofibers were found to have diameters from 500 nm – 2 μm which made them small enough to provide a high surface area to volume ratio capable of increasing the number of peptide substrates we could bind in a given area but also providing support for the flow-through applications we intend to use them for. Smaller nanofibers were much more brittle and lacked the uniformity as fibers in the 1 μm range.

Porous surfaces can have display tremendous surface areas capable of vastly increased attachment sites for biomolecules. Problems arise when these surface don't have pore sizes large enough for the biomolecule to enter the porous matrix. Although the peptide substrates are very small some biomolecular sensing mechanisms are larger and might not be able to function in small pores. Also higher molecular weight analytes might not be able to reach the sensing system and steric hindrance might prevent the mechanism form operating correctly.

The nanofibers were very easily produced and maintained a fairly constant morphology. The optimized solution and spinning parameters were found previously and the final protocol involved using 1.0 g PMMA in 10 ml acetone and spinning at 20 kV, 5

ml/hr, with an 18 cm separation distance. Other factors include the atmospheric conditions which very slightly affected our results since our chamber is kept in a temperature controlled environment. The functionalization method involving hexamethylene diamine was selected for its simplicity and its capability to produce uniform and accessible aminated surfaces. These aminated surfaces were then easily linked to the peptide substrate using a simple cross-linking method. The immobilization process could also be adapted to bind other biorecognition molecules.

The peptide substrate sensing mechanism contained fluorophores on each side that interacted with each other by transferring energy from the donor to acceptor when excited and when in close proximity (Grahn et al. 1998). This FRET system has been used before and has been considered to be one of the best methods for protease analysis and characterization (Ellard et al. 2002). Other proteases have been studied on different platforms and further research should identify other candidates using this sensing method. These include anthrax and botulinum to name a few biothreat agents that are of most concern for homeland security officials (Williams et al. 2003). Methods to develop peptide substrates that are very specific to an analtye involve using phage display libraries. Simple, hardy ligands are found that bind to target species for detection purposes. These probes are inexpensive and can be used for portable deployment of the sensing system.

The portable optical detection system was tested and results were inconclusive. Low grade components and samples optimized for the fluorescence microscope hindered the ability to truly test this system.

The response of our design showed a limit of detection down to 100 pM. This was much better than our in-solution testing results and is orders of magnitude greater than our previous designs involving nanoparticles. We have achieved in developing a sensor system that can be used for flow-through detection of a potential analyte. This design could be further developed to be portable and expanded to other applications and analytes. The sensor system was easy to fabricate and is very inexpensive to produce. Although our design does not support all aspects of an ideal biosensor, we believe that future tweaking and design changes can lead to a very high performance biosensor.

7.5 Conclusion

Our results indicate that immobilizing peptides onto nanofibers provides a viable method for protease detection. There was a fluorescence response upon addition of the enzymes to the biosensor after 5 minutes and the dosage response study resulted in a limit of detection in the range of 100 pM for trypsin. The increased surface area and 3-dimensional interaction of the analyte with the sensing surface aided in developing a flow-through sensor with a lower limit of detection and a fast response. We hope to continue to develop this sensing system and put it to use in field deployable applications.

Acknowledgements

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Chapter 8

CONCLUSION

The research presented is an investigation into nanomaterial platforms and peptides substrates for the design of a protease biosensor. Essentially 4 nanoplatforms were analyzed and appropriate sensing mechanisms were utilized to determine the sensitivity and response time. Nanofiber platforms seem to hold the most promise for a commercialized biosensor in the detection of protease analytes. Results showed that upon the addition of protease, the peptides cleaved resulting in detectable optical changes due to the distance-dependent change between the fluorescent molecules. Optimization of the sensing system can always be further tested.

The results show that the optical-based peptide substrate biosensor can become a useful analytical tool for detection of protease. Also the feasibility of the custom-built optical detection system for detection was demonstrated. This is portable and would permit on-site analysis of samples, reducing the need for lab testing. It is also sensitive and rapid and could be applied across many applications.

Chapter 9

FUTURE WORK

A wide range of sensing platforms have been investigated in this dissertation. Trypsin and thrombin were the main two analytes of interest, and mainly trypsin due to its availability and activity to demonstrate the sensor function. Further research could investigate different analytes that act in the same way as trypsin (i.e. cleaving a substrate). After investigation of these analytes it has been found that botulinum neurotoxin, hepatitis C virus, and HIV protease are a few possibilities (Sapsford et al. 2008b). Other biological threat agents, such as tri-nitro-toluene (TNT), Anthrax, Tetanus, and Sarin Gas could also be targeted (Iqbal et al. 2000; Rowe-Taitt et al. 2000). Our investigation with detecting botulinum neurotoxin was initially unproductive due to the condition requirements of the neurotoxin to function. Exact buffer solutions, longer peptides, and temperature requirements were needed to be controlled for this assay. Some of the peptide sequences have been found for the above analytes and many new designs for peptide substrates could be found investigating other systems and using phage display libraries (Goldman et al. 2000; Vikholm-Lundin et al. 2008).

The nanofiber sensing platform could also be investigated further coupling it with flow through detection equipment (microfluidics and air samplers) to design a remotely deployable sensing unit. Using devices to pre-concentrate samples could help in detection of the smallest traces of an analtye. Working with other engineering disciplines, including electrical, computer, chemical and mechanical engineers, and the medical school could

prove beneficial in designing the next bed-side monitoring device or remotely deployable biothreat detection system.

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Vita

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