Development of a Quantum Dot Based Microcapillary

Immunosensor for Detection of Biomarkers

A Thesis

Submitted to the Faculty

of

Drexel University

by

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in partial fulfillment of the

requirements for the degree

of

Master of Science in Biomedical Engineering

February 2009
Acknowledgement

First and foremost, I would like to thank Dr. Elisabeth Papazoglou (School of Biomedical Engineering, Drexel University, Philadelphia, PA) and Dr. Sreekant Murthy (College of Medicine, Drexel University) for their excellent guidance, support and patience during the course of this work. Under their guidance I was presented with challenges that gave valuable learning experience. I would also like to thank Dr. Andres Kriete for being in the committee and Dr. Peter Lelkes for allowing me to use his lab facilities.

I owe my sincere thanks to Dr. Nadarajan Sundar Babu (School of Biomedical Engineering, Drexel University, Philadelphia, PA) for his inputs on various aspects of the project, Dr. Leonid Zubkov (School of Biomedical Engineering, Drexel University, Philadelphia, PA) for his guidance in developing the optical set up and Mr. Vishal Kamat (School of Biomedical Engineering, Drexel University, Philadelphia, PA) for teaching me various bioconjugation techniques. I also thank Mr. Kalyan Chakravarthy for providing me the animal stool samples.

I would like to thank administrative staff of School of Biomedical Engineering at Drexel University and my colleagues Mr. Mike Neidrauer, Mr. Peter Clarke and Ms. Chetana Sunkari. I would also like to thank my friends Mr. Gaurav Gandhi, Mr. Sandeep Banerjee, Mr. Sameet Shriyan, Mr. Srijak Bhatnagar, Mr. Johann Desa, Mr. Ertan Ergezen and Mr. Agastya Anishetty.
Lastly, I thank my parents and family members for their continuous support and encouragement through these years.
Dedication

I dedicate this thesis to my parents for their unconditional love and support. Without their patience and understanding none of this would have been possible.
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Abstract

The unique properties of Quantum Dots (QDs) such as broad excitation spectra, narrow emission spectra, high resistance to photobleaching, higher photo-stability and stronger fluorescence makes them one of the most attractive fluorescent reporter molecules for a wide range of biological applications including biosensing and assay development applications. However, the high cost of QDs has limited their application in assay development where at least 50-100µL reagents are required for performing an assay. This research capitalizes on the superior properties of QDs by utilizing just 1-2µL of sample and antibodies conjugated to QDs for detection of biomarkers. In this work we developed a simple and inexpensive microcapillary immunoassay based on quantum dot assay for detecting Myeloperoxidase (MPO) in animal stool samples. The method utilizes commercially available polymethylmethaacrylate (PMMA) micro-capillaries (I.D. 250µm, O.D. 500µm) as substrates for performing a sandwich assay. The results obtained indicate that the limit of detection (LOD) of the device and the assay is 100 picomolar of MPO (~15 ng/mL) in 1-2µL of sample. The specificity of the assay was tested in a solution mixture containing MPO and Matrix Metalloproteinase 1 (MMP 1). Furthermore, the assay and device robustness was tested by detecting MPO in animal stool samples and comparing the results obtained with a commercially available MPO ELISA kit which uses 100µL of sample.
1. Introduction to Biosensors

1.1 Overview

A sensor in its true sense is a bridge in the gulf between stimuli and the response. Biological Sensors are those conduits which decode the information sent by one system of the body and communicate with another system to react to the information. Variant examples spread over glucose production, reflex motion, and hormone secretion in interaction with the external environment. A biosensor is in effect an artificial sensor with a biological component attached intermittently to mimic the action of a naturally evolved algorithm. The most common example of a sensor is a sensory organ such as the nose and an example of a manmade sensor is the litmus paper for measuring pH. A sensor primarily comprises of 3 components: a recognition unit, a transduction unit and a signal processing unit. The recognition component detects the presence of the substance to be measured, called the analyte. Once the analyte is detected the transduction unit or transducer, transforms the interaction of the recognition unit and the analyte to a measurable/quantifiable signal. The signal from the transducer is converted to a meaningful result by the signal processing unit. Figure 1 shows the basic components of a sensor.
In a sensor, the signal produced can be transduced in various forms. The usual forms include thermal, electrical and optical signals. In the case of thermal transducers, a change in temperature is measured. Electrical transducers can measure a change in voltage or in the number of electrons (current) corresponding to different responses. In optical transducers, a change in the number of photons is measured to assess the sensor’s response.

A device which integrates a biological element on to assess a specific interaction with a signal transducer is called a **Biosensor**. In a biosensor, the recognition unit is usually a biological component such as an Antibody, an Antigen, an Enzyme or an array of cells. The use of these biological components makes the sensors specific for a particular analyte/substance. These types of sensors are often referred to as Affinity Sensors.

![Figure 1: Schematic Representation of the Components of a Sensor](image-url)
A subclass of affinity sensors is that of immunosensors, which use immunological principles for detection purposes. In immunosensors, specific antibody / antigen interactions are exploited. The specificity and affinity of antibody-antigen interaction has allowed researchers to develop assays for a particular biomarker of interest.

Antibodies are gamma globulin proteins produced in the bodies of vertebrates in response to an alien stimulus. An alien stimulus can be any foreign body which in biological parlance is called an antigen. Antibodies have a fascinating property of combining with the antigens which have triggered their production. Antibodies are produced by the cells of the immune system called the B-Cells. Hence, these antibodies protect our body from various microbes, infections and help fight various diseases. When the immune system
recognizes an antigen it induces a response which converts the B-Cells into plasma cells. These plasma cells secrete antibodies into the bloodstream to help eliminate the antigens. The antibody which is usually secreted in response to an antigen is Immunoglobulin G (IgG). The structure of the antibody is shown in Figure 3. All antibodies have a specific binding site for an antigen and this binding site varies between antibodies produced in response to antigens. The presence of the antigen binding site (epitope) makes the antibody-antigen reaction highly specific. This specificity is utilized in immunosensors.

Figure 3: Structure of Antibody (Adapted from Immunology by Kuby)
In an immunosensor, various assay methodologies can be used to detect the antigen. For e.g., direct immunosensors, competitive immunosensors, sandwich immunosensors, displacement immunosensors etc\(^4\). Figure 4 shows the different types of immunosensors mechanisms. Our research is focused on developing an optical biosensor for detection of Myeloperoxidase (MPO). The work primarily focused on developing a sandwich micro-immunosensor utilizing fluorescent nano-particles (Quantum Dots) as the reporting moieties.

Figure 4: Various assay formats used in immunosensors\(^4\).
1.1.1 Optical Biosensors

Optical biosensors transduce optical signals resulting from biological interactions\textsuperscript{6}. The optical signal produced is directly proportional to the concentration of the biological or chemical analyte\textsuperscript{7}. The past decade or so has seen an exponential increase in the number and range of available biosensors based on different optical phenomena. Optical biosensors are based on different types of optical phenomena, for example, electrochemiluminescence, fluorescence, interferometry, phosphorescence, absorption, planar waveguides, evanescent waves, surface plasmon resonance (SPR), etc\textsuperscript{6}. Recent advances in instrumentation combined with breakthroughs in experimental design have led to the increasing application of optical biosensors in many areas\textsuperscript{6, 8}.

Optical fibers were the first optical transducers which were used to develop a biosensor. Optical fibers act as waveguides for the transmission of optical signals\textsuperscript{6}. One of the first optical fiber-based sensors was described about 25 years ago by Freeman and Seitz\textsuperscript{9}. Briefly, the sensor system used a 2 feet-long optical fiber with a 1/8 inch diameter fiber optic cable. One end of the cable was used to carry out the chemiluminescence reaction and the other end was used to detect the chemiluminescence signal resulting from the conversion of peroxide to peroxidase by a photomultiplier tube (PMT)\textsuperscript{10}.

After the advent of the first optical fiber biosensor, many different optical fiber biosensors have been developed for different areas of research such as environmental\textsuperscript{11, 12}, clinical\textsuperscript{13, 14}, detection of heavy metals\textsuperscript{15, 16} etc. Salama et al reported development of an optical fiber-based chemiluminescent immunoassay for the detection of autoimmune
antibodies against ovarian and breast cancer-associated antigens. The group successfully detected antibodies against GIPC-1, a protein involved in regulation of G-Protein signaling. The research primarily focused on the development of an immunosensor for detecting human monoclonal 27.B IgM in patients suffering from ovarian/breast cancer. The immunosensor’s Limit of Detection (LOD) was 30 pg/mL, which is 50 times lower than the traditional 96 well ELISA assay.\textsuperscript{13}

Another class of optical biosensors which has been used widely are Surface Plasmon Resonance (SPR) sensors. SPR sensors measure the refractive index changes occurring at the surface of a metal film supporting a surface plasmon.\textsuperscript{17} The most popular SPR system is the BIAcore\textsuperscript{TM} instrument from Biacore\textsuperscript{3}. The BIAcore\textsuperscript{TM} instrument has been used to study the specificity, kinetics, and binding affinity of antibody-antigen reactions.

Two other popular categories of optical biosensors include capillary based optical biosensors and microfluidic based optical biosensors. Both these systems use micro level of reagent volumes. Hence, these two categories of sensors can be termed as “Optical Micro-Immunosensors”. The focus of this research is the development of a capillary optical immunosensor utilizing fluorescence for the detection of an antigen analyte.

\textbf{1.1.1.1 Microfluidic Immunosensors}

Microfluidic immunosensors comprise of micron level channels where the immunoassay reaction takes place. The most common assays used are sandwich assays followed by competitive assays for small molecules.\textsuperscript{18} The primary components in microfluidic devices are the micro-channels. These micro-channels reduce the volume of reagents to
be used to only a few micro liters. Microfluidic based immunosensors offer several advantages over their 96 well plate counterparts. The advantages are listed below\textsuperscript{18-20}.

1. **Less Reagents and Smaller Sample Volumes:** Micro-channels, the primary components in microfluidic devices enable the user to perform immunoassays by using volumes less than 10 micro-liters. This reduction in the use of reagents and samples results in significant cost reduction because most of the antibodies, fluorescent molecules and other reagents for immunoassay are very costly.

2. **Reduced Analysis Time:** The small dimensions of micro-channels reduce the diffusion distance between the antibody immobilized on the solid substrate and the antigen present in the liquid phase. The reduction in diffusion distance results in the reduction of antibody-antigen reaction time. Hence, this can reduce the analysis time from a few hours to a few minutes.

3. **Automation and Integration:** Microfluidic devices can be made fully automated by using proper pumps and valves to transport the liquid. This automation helps to reduce errors which are inherent in lab based bench top immunoassays.

Integration of small reagent storage chambers into microfluidic devices allows them to be used as point of care diagnostic devices. The small volumes of liquids in a biochip can be moved by capillary actions, thus making the device portable, automated and easy to use\textsuperscript{19}. 
Owing to their advantages microfluidic devices have been used widely for biological sensing purposes and have been successfully integrated with optical components\(^{20}\). Current applications include the analysis of clinically relevant biomarkers such as C-reactive protein (CRP)\(^{21}\), interleukin-6 (IL-6)\(^{22}, \ 23\), prostate specific antigen (PSA)\(^{24}\) and others.

Klostranec et al.\(^{25}\) successfully integrated microfluidics, optics and use of quantum dots in their work. They demonstrated the superior performance of their device by detecting blood-borne infectious disease markers in blood serum. They were able to detect markers for Hepatitis C, B and HIV. The sample volume used was less than 100µL and the assay time was less than one hour. In this research work, the biomarkers (antigens) are covalently attached to the surface of the Qdots and subsequent sandwich assay was performed by incubation with alexa fluor 488 conjugated antibodies. This was followed by passing the Qdot complex through the microfluidic channel exposed to a laser spot (488nm and 25mW). The fluorescence signal generated was collected by a 60x oil immersion objective with a numerical aperture of 1.35. The collected signal is passed on to a solid state photodetector where a change in the voltage is measured to check the presence of the antigen. Even though the limit of detection of this system was \(10^{-12}\) M, the system used expensive optical components and photodetectors to detect the presence of antigens. In our research work, we report an inexpensive optical set up utilizing UV LEDs and a monochrome CCD camera with a limit of detection of 100 picomolar.
Diercks et al\textsuperscript{26} reported development of a microfluidic device which can detect multiple protein markers in a sensitive and quantitative way in nano-liter volumes of sample by using optically encoded microsphere beads.

The success of microfluidic immunosensors in research labs has been translated into commercial products with ease. One such example is \textit{“Triage Meter”} from Biosite Incorporation. A fluorescent probe is used for detection of various cardiac markers, toxicity test and drug abuse test. A 5 milliwatt laser is used to excite the fluorophores and a silicone photodiode is used to detect the fluorescence signal. Figure 5 below shows a picture of the Triage Meter, composed of two primary components: the microfluidic Chips and a read out unit\textsuperscript{27}.

\textbf{Figure 5}: Triage Meter Panel (Adapted From Biosite Inc Website)\textsuperscript{27}
1.1.1.2 Capillary Biosensors

Capillaries have found extensive application in numerous disciplines of science, ranging from electronics to semiconductors, from manufacturing to biological sampling & detection methods, like chromatography\(^{28}\). The versatility of a capillary comes from its geometry, its high surface area to volume ratio, possibility to use a transparent construction material and create a surface with perfect adhesion\(^{29}\).

A sturdy minuscule capillary construction allows the immunosensor device to be compact and portable; the small size ensures easy and accurate detection even for very small samples size, with combined cost - effectiveness because the small sample reduces the amount of expensive reagents required. A larger surface to volume ratio in a capillary compared to the geometry of wells in well-plates (currently used as standards), can accelerate the detection reactions and increase sensitivity, by reducing the role of diffusion\(^{30}\). Capillary based biosensors can eliminate the complex process of microfabrication required for microfluidic immunosensors. It is all these characteristics that make capillary an excellent choice for construction of a cost-effective immunoassay device.

Capillary tubes can act as both optical waveguide and sampling chambers. The cylindrical shape facilitates easy and uninterrupted flow-in and flow-out of samples and the inner surface can be used for immobilization of probing molecules such as antibodies\(^{31}\).
Both glass and polymer based capillaries have been used to carry out immunoassays. Specifically glass or fused silica\textsuperscript{18, 32-34}, polystyrene\textsuperscript{35}, polymethylpentene\textsuperscript{36}, polymethylmethacrylate\textsuperscript{37}, poly-vinyl chloride\textsuperscript{38} have been used in fabricating capillary biosensors. Polymeric tubes may pose a manufacturing challenge and glass capillary tubes need additional processing steps of surface activation for successful fixation and immobilization of probes.

The inert nature of glass makes it necessary to perform additional chemical modification to form covalent bonds between a glass surface and a biomolecule. Chemicals used for such modification / functionalization comprise of silinazing agents like 3-mercaptopropyl trimethoxysilane. These silanizing agents bind simultaneously to the hydroxy groups on the glass silicate surface and to various groups on the probe surface ranging from the amino terminal to, thiol groups, based upon the specific silanizing agent\textsuperscript{34}. Therefore, the choice of these silinazing agents depends upon the type of immunoassay that they are going to be used in and the probes that they need to attach to.

On the other hand, polymeric capillaries are of particular interest due to readily available functional groups on their surface offering an appropriate substrate for immobilizing antibodies or antigens, without special modification steps. Furthermore, recent developments in photochemical methods of functionalizing polymeric materials provide compelling reasons to choose polymeric capillaries over fused silica or glass capillaries\textsuperscript{39, 40}. 
Several strategies have been devised to detect low concentrations of antigens by solid phase immunoassay in capillaries, primarily focusing on excitation of fluorophores followed by collection of the emitted photons. One such approach takes advantage of the evanescent field at the interface of the polymer/liquid interface for collecting the emitted signal; this particular method requires the material of the capillary to function as a waveguide\textsuperscript{28}.

The first capillary waveguide application for a sensor was reported in 1994 by Weigl and Wolfbeis\textsuperscript{41}. The chemical sensor measured carbon dioxide. Substances which produce a color change when they come in contact with carbon dioxide were immobilized on the inner surface of the capillary. Optical fibers were used for both excitation and collection. Both the excitation and collection fiber were perpendicular to the capillary.

In 2002, Ligler et al\textsuperscript{42} provided a summary on four different potential excitation and collection methods for integrating waveguide capillaries.
One of the techniques (Figure 6, Method 3) is analogous to methods used in this research project. An evanescent wave is used for excitation of bound fluorophores. Fluorescent emission can be collected at the end of the capillary by propagating it down the axis (Figure 6, Method 2 and 4). Alternatively, a detector can be placed perpendicular to the axis of the capillary for data collection (Figure 6, Method 3). The later method was used in this research to detect fluorescence from the capillary.
Several biosensors utilize the integrating waveguide capillary biosensor design with data collection from the ends of the capillary. Zhu et al reported development of a capillary based waveguide sensor for detection of E. coli O157\(^{43}\). The method described utilized a capillary based sandwich immunoassay for detecting the presence of E. coli O157. In this work, the signal from the capillary is integrated over the length of the capillary and the signal was detected at one end of the capillary (Figure 6, Method 4). The volume of sample used in this case was 75µL.

Recently, Stringer et al\(^{44}\) reported a capillary biosensor based on Forster Resonance Energy Transfer (FRET). The sensor utilized the superior properties of Quantum Dot Fluorescent particles and a Teflon AF capillary as a Liquid Core Waveguide to detect the presence of Troponin I in solution as well as in biological samples. This work used a laser system for excitation of the fluorophores and used the capillaries as integrated waveguide demonstrated in Method 4 of Figure 6. The limit of detection of the sensor was 32nM in solution for troponin I and 55nM in spiked human plasma.

In this work we are reporting a low cost PMMA microcapillary biosensor using Quantum Dots as the fluorescent probes for detection of picomolar quantities of analytes, and demonstrating this capability by detecting Myeloperoxidase (MPO) in solutions and animal samples. Our selection of PMMA was based on its optical properties and the capability to selectively functionalize its surface for covalently immobilizing antibodies.

1.2 Fluorescence
Fluorescence is widely employed in biology for both imaging and detection assays. Advances in optics hardware and availability of more sensitive fluorescent probes for conjugation to antibodies and antigens have enabled continuous improvement in fluorescence based imaging and detection. The molecules which exhibit the phenomenon of fluorescence are called fluorophores. Most fluorophores are organic molecules. These molecules absorb light at a certain wavelength, resulting in excitation of an electron. The excited electron moves to a higher energy level from the ground state. The excited electron starts emitting photons while returning back to the ground state. The emission of this photon results in fluorescence\(^45\). The Jablonski diagram (Figure 7) depicts the process of fluorescence\(^46\).

![Jablonski diagram](image)

**Figure 7**: Simplified Jablonski diagram depicting the phenomena of fluorescence \(^46\)
Examples of organic fluorophores include fluorescein, rhodamine, Alexa Fluor. Despite their wide usage they pose several challenges. For example, the organic dyes photobleach in confocal laser scanning microscopy, because of the high power laser used for excitation. Other disadvantages include poor photo stability, narrow absorption spectra and broad emission spectra. Owing to these issues the organic dyes pose limitations in their usage for quantitative biological detection, such as in sensitive assays. Semiconductor based fluorophores such as the Quantum Dots (QDs) offer an attractive alternative.

1.2.1 Quantum Dots

The advent of advanced engineering capabilities in areas exploring complex and highly intelligent facets of biology has opened big vistas into understanding the core architecture and working of science. Nanotechnology is one of the greatest advances of modern science exploring the same. One key application of nanotechnology is the development of luminescent nano sized particles. The most popular fluorescent nanoparticles for biological application are Quantum Dots\(^{47}\). At nanometer scale these charged carriers (electrons/holes) exhibit quantum confinement effect at room temperature and give rise to material properties that not only differ from their bulk form but can also be fine tuned by controlling their quantum yield\(^{48}\). The size of the nano-particle controls the emission spectrum, while UV light excites all quantum dots. Hence, by selecting the size of the particles, QDs with different emission spectra can be synthesized\(^{47}\).
QDs exhibit several advantages over organic fluorophores. These advantages include broad excitation spectra stretching into the ultraviolet (UV) region, narrow emission spectra, large Stokes Shift, high resistance to photobleaching, higher photo-stability and stronger fluorescence\(^{49}\).

Multiplexing, although an attractive way to detect multiple markers, has remained a challenge in organic fluorophores due to bleed through in the emission bands and the requirement of multiple excitation sources. Solutions include expensive hardware and software combinations such as those used in expensive Flow Cytometry Instruments (FACS). On the contrary, the broad excitation spectra in QDs allow multiplexing with Quantum Dots with a single excitation source and their narrow emission spectra address the issue of overlapping emission spectra which existent is organic fluorophores\(^{50}\).

Figure 8 (adapted from the Invitrogen website) shows the emission spectra of different QDs\(^{51}\).

Extreme level of resistance to photobleaching allows QDs to be employed in various systems where high power excitation sources are used, for e.g., confocal laser scanning microscopy. It has been reported that QDs exhibit 100 times more stability to photobleaching than their organic fluorophores counter parts\(^{52}\).

This higher photo-stability of QDs is crucial for experiments where continuous fluorescence measurements are required, for example in live cell imaging for monitoring particular cellular trafficking events\(^{53}\). The stronger and brighter fluorescence properties
of QDs help in overcoming the auto-fluorescence signal that is particularly high in the *in vivo* conditions\textsuperscript{52, 54}.

QDs are semiconductor nanoparticles which comprise of a core shell structure. Two of the most widely used commercial QDs come with a core of CdSe or CdTe and a shell of ZnS and emissions from 405nm to 805nm\textsuperscript{50, 52}. The shell stabilizes the structure, helps to overcome quenching compared to a QD made only from a core and provides a large surface area available for further modification.

**Figure 8**: Spectra of QDs with different emission wavelength with same excitation wavelength of 405nm.

*L to R: QD525, QD565, QD605, QD655, QD700. The numbers denote the emission wavelength (Adapted from Invitrogen Website\textsuperscript{51}).*
In order to make QDs suitable for biological imaging and use in a biological environment they also have to be rendered water soluble. This is done by capping the shell with a polymer layer that contains a hydrophobic segment facing inside towards the shell and a hydrophilic segment facing outside. The hydrophilic layer can be modified to include functional groups such as –COOH and –NH₂ groups for further conjugation to proteins and antibodies or oligonucleotides⁵²,⁵³,⁵⁵.
QD conjugation to biomolecules is achieved by different ways like electrostatic binding, non covalent biotin-streptavidin bonding or covalent bonding. Figure 9 shows the different ways of conjugating biomolecules to QDs. The most widely used conjugation technique of all is the covalent bond formation between the QDs surface and biomolecules. Surface modifications on QDs allow easier covalent bond formation. In one of the most widely used methods, amine terminated QDs are used for conjugating antibodies. The amine terminated QDs are activated with a malemide containing crosslinker molecule which can be then conjugated to a fragment or whole antibody molecule. Some of the most commonly employed QD conjugation methods are based on...
crosslinking reactions between amine and sulfhydryl groups, between carboxylic acid and amine group, between aldehyde and hydrazide functions. The carboxylic-amine bond has one advantage over all other methods; this method doesn't require any modification of the antibody before QD conjugation. In case of amine and sulfhydryl bond formation, the antibody should be reduced to expose their interchain -SH bonds. In the case of aldehyde and hydrazide bonds, carbohydrate groups on the Fc portion of the antibody are oxidized. These modifications on antibodies may affect their performance to a certain extent. Xing et al compared the performance of various QD-conjugation methods for immunohistochemical staining. Table 1 shows the summary of properties and performance of the different QD-Ab conjugates they evaluated for immunohistochemical staining.
**Figure 11**: Schematic showing different bioconjugation methods used to conjugate antibodies/proteins to QDs\textsuperscript{56}
In this research sulfhydryl conjugation method was used. The reason for using this conjugation method was mainly due to its availability in the market and medium cost. Applications of QDs encompass many areas including single cell labeling and imaging.

Table 1: Characteristics and performance of different QD-Ab conjugation methods

<table>
<thead>
<tr>
<th>Conjugation Method</th>
<th>Sulfhydryl</th>
<th>Amide</th>
<th>Fe-Sugar</th>
<th>Biotin-Avidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond Type</td>
<td>Covalent</td>
<td>Covalent</td>
<td>Covalent</td>
<td>Non-covalent</td>
</tr>
<tr>
<td>Conjugated Ligand</td>
<td>Ab fragments</td>
<td>Whole Ab</td>
<td>Whole Ab</td>
<td>Whole Ab</td>
</tr>
<tr>
<td>Site Specificity</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ligand Orientation</td>
<td>Fixed</td>
<td>Random</td>
<td>Fixed</td>
<td>Both depending on biotinylation condition</td>
</tr>
<tr>
<td>Ab/QD ratio</td>
<td>~4</td>
<td>~15</td>
<td>~15</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>Background Noise</td>
<td>Low</td>
<td>Medium</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Reagent Costs</td>
<td>Medium</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>Overall Performance</td>
<td>Fair</td>
<td>Poor</td>
<td>Excellent</td>
<td>Good</td>
</tr>
</tbody>
</table>

In this research sulfhydryl conjugation method was used. The reason for using this conjugation method was mainly due to its availability in the market and medium cost. Applications of QDs encompass many areas including single cell labeling and imaging,
imaging of signaling pathways, deep tissue imaging, *in vivo* tumor and cancer imaging, immunosensor development, assay labeling as well as their use as FRET donors\(^57\).

Kerman et al, reported a QD based immunosensor for the detection of prostate-specific antigen\(^58\). A disposable substrate with carbon on surface was used to carry out a sandwich assay. Primary antibody was immobilized on the substrate surface and the antigen was captured by a biotinylated secondary Ab. The detection was done by introducing Streptavidin QD. The limit of detection of this immunosensor in serum was 0.25 ng/mL. QDs have also been used in protein microarrays for early detection of cancer. In one such application, Zajac et al reported detection of TNF, IL-6, IL-8, MIP-1\(\beta\), IL-13 and IL-1\(\beta\) at picomolar concentrations\(^59\).

Quantum dots (QDs) have also found significant applications in biology especially in live cell imaging\(^60\) and to follow and understand signaling pathways\(^61\). Due to their excellent properties QDs were selected in this project for developing a capillary based immunosensor.
2. RESEARCH OBJECTIVE

The objective of this research was to develop a low cost Polymer Microcapillary biosensor using QDs as the fluorescent reporting molecules. As mentioned earlier, current organic dyes have many limitations when it comes to biosensors and multiplexing applications. In order to overcome these limitations, QDs were used in this research. The main disadvantage of QDs is their high cost. In order to address this issue, we developed a “polymer microcapillary” sensor using just 1-2 µL of sample and QD-Ab solution. PMMA was selected for the capillary material because of its superior optical properties and ease of surface modification for antibody/antigen immobilization on its surface. Several optical sensing methods were evaluated during the project and compared for detecting bioanalytes. The detection capability of the sensor was demonstrated by using myeloperoxidase (MPO), an inflammatory marker over-expressed in all inflammatory diseases including those of the gastrointestinal tract. Desired capabilities of the biosensor include fast, accurate and reliable detection of MPO and inexpensive optical set up for detection. In this research, Light Emitting Diodes (LEDs) were used, which are a significantly less expensive than LASER based systems.

The goals of this research can be summarized as:

- Demonstrate detection of a bioanalyte, MPO in this case, at picomolar concentrations using 1-2µL volume of sample.
• Develop a sandwich immunoassay using Quantum Dots as fluorescent reporting molecules.

• Develop an inexpensive optical detection system for reliable fluorescence detection.

• Demonstrate the ability to detect MPO in biological samples, specifically animal stool samples of a disease simulating human IBD.

A key reason for selecting MPO to demonstrate the performance of the capillary microimmunosensor is the availability of an animal model where inflammation can be induced to produce MPO in animal stool samples. This model has been in use by Dr. Murthy for years\textsuperscript{62} and the ability to detect MPO in tissue samples of diseased animals through QD technology has been previously demonstrated by our research group\textsuperscript{63}.

The final goal of this project is to develop a fully automated multi-microcapillary set up for fast and reliable detection of biomarkers. The research work presented here forms the basic foundation of this goal.
3. Myeloperoxidase

Introduction

Myeloperoxidase (MPO) is a peroxidase enzyme present in neutrophil granulocytes. It is an abundant leukocytic enzyme and the heme segment of its structure gives it its greenish characteristic color. It is a liposomal protein seen in neutrophils rich body secretions such as pus and mucus. It is also found in minute quantities in macrophages and monocytes.

Production

MPO is expressed by a 14kb gene which consists of 11 introns and 12 exons. The gene is located on the long arm of chromosome 17 in the segment q12-24. A 80 kDa preproMPO is the first product of translation. This preproMPO is processed the endoplasmic reticulum to produce active proMPO. The proMPO is exported into the Golgi compartment. This propeptide is processed in the azurophilic granules.

Structure

MPO is a heme enzyme of two dimers each of which contains heme prosthetic groups with equal reactivity to Hydrogen peroxide. MPO is known to be strongly cationic. MPO is a 146 kDa dimer connected by a single disulphide bridge between each unit. Each unit of the dimer consists of two polypeptides, one light chain (108 amino acids;
14.5 kDa) and one heavy chain (466 amino acids; 58.5 kDa)\textsuperscript{66}. Figure 12 shows the 3D structure of Human Myeloperoxidase with heme complex\textsuperscript{68}. Three isoforms of MPO are in prevalence, and they vary only in the size of the heavy chains\textsuperscript{66, 69}.

![Figure 12: Structure of Human Myeloperoxidase Complex with Heme Complex\textsuperscript{68}]

**Current Relevance**

MPO plays a primary role in the immune response during the inflammation caused by the protective ring of the host’s defense mechanism. High levels of MPO in serum have been related to higher risk of coronary disease occurrence\textsuperscript{70}. MPO has been shown to be present


in the atherosclerotic lesions. The MPO/H2O2/HOCl system along with the nitric oxide is responsible for the oxidation of low density lipoprotein (LDL). This modified LDL is uptaken by macrophages resulting in the formation of foam cells. Modified LDL also interferes with the biological activity of nitric oxide (NO) as a vasodilator triggering platelet activation.

Elevated level of MPO has been found in stool samples of patients suffering from various forms of Inflammatory Bowel Disease like ulcerative colitis and Crohn’s disease.

In this research work MPO was used to demonstrate the functionality of a QD based microcapillary sensor and complements the work of Karwa et al who demonstrated the presence of MPO in the colitis tissue of an animal model and the correlation of MPO expression to inflammation and disease progression. Stool samples obtained from the same animal (mouse) model were used in this work to demonstrate the capability of the QD based microcapillary sensor to detect MPO ex-vivo.
4. MATERIALS AND METHODS

4.1 Materials

The capillaries used in this study were obtained from Paradigm Optics Inc (O.D. 500 microns, I.D. 250 microns). In order to prevent “buckling” of the polymer capillaries and be able to hold them straight, we used in the beginning of our studies glass capillaries from World Precision Instruments, Sarasota, FL, USA. Later, a customized system based on spring loaded system was developed to hold the capillary straight. Rabbit anti Human polyclonal MPO antibody was purchased from ABD Serotec, Raleigh, NC, USA and this antibody is used to coat the polymer surface. Zero length cross linkers 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Pierce Biotechnology Inc., IL, Rockford, USA. Monoclonal anti – Human MPO antibody and antigen MPO were obtained from LEE Biosolutions, St. Louis, Missouri, USA. Fetal Bovine Serum (FBS), 10X Phosphate Buffer Saline, Sodium Azide, Peristaltic Pump and its accessories were obtained from Fisher Scientific, Pittsburg, PA. EDTA, Tween 20, Protease Inhibitor and Glycerol were obtained from Sigma Aldrich. Quantum Dot Conjugation Kit was obtained from Invitrogen Corporation, Fredrick, Maryland. The Syringe Pump was obtained from KD Scientific Holliston, MA. UV curable glue was obtained from Edmund Optics, Barrington, NJ.

The animal stool samples were provided by Mr. Kalyan Chakravarthy and Dr. Sreekant Murthy.
4.1.1 Optical Components

For excitation of the QDs UV-LEDs with two different output power were obtained from Nichia Corp. The UV-LEDs used had optical output power of 10 mW and 300 mW respectively. A long pass filter and a band pass filter (600±20 nm) were obtained from Edmund Optics, Barrington, NJ. A monochrome CCD camera (COHU 4900) was purchased from Cohu Inc., San Diego, CA and a firewire monochrome CCD camera (Stingray, AVT-FS-033B) purchased from 1stVision Incorporated, Andover, MA.
4.2 Methods

4.2.1. Quantum Dot Conjugation

Quantum Dots were conjugated to antibodies according to the protocol provided by Invitrogen Corporation. Briefly, the amine terminated QDs were activated by a heterobifunctional crosslinker 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC). The antibodies were reduced using DTT (Dithiothreitol) exposing the thiol groups (-SH) from the broken disulfide bridges. The activated QDs and the reduced antibody fragments were then brought to react to form the conjugate. Unconjugated antibodies and QDs were removed from the conjugated QD-Abs by running the whole mixture through a separation column. For each conjugation, 125µL of 8µM amine terminated QDs and 300µL of 1mg/mL monoclonal antibody was used. Each conjugation procedure yields approximately 200µL of conjugates of approximately 1µM concentration.
4.2.2 Capillary Functionalization and MPO Assay

Functionalization of PMMA capillaries (pCaps) was carried out by following an alkaline ester hydrolysis of methacrylate, a method adapted from Bai et al. for functionalizing PMMA \textsuperscript{75}. The method was modified slightly, 1N NaOH at 60°C was pumped through the pCaps using a peristaltic pump at 100µL/min for one hour followed by washing with 1X PBS buffer (pH 7.4). This step hydrolyzes the acrylate ester group on the surface of the pCAP resulting in COOH termination. The availability of COOH functional groups is utilized to covalently bind the MPO polyclonal antibody to the inner walls of the capillary. Functionalized pCAPs were then treated with EDC/NHS solution (104.7mM EDC 21.7mM NHS) in PBS pH 6.0 for 5 hours\textsuperscript{40}. The activation was followed by removal of excess EDC/NHS by washing the pCaps with 1X PBS pH 7.4. After this, the capillaries were loaded with 100nM MPO polyclonal antibody. Optimal immobilization of the polyclonal MPO antibody on the inner surface of the pCAP was accomplished by incubation at 4°C for 16 hours. Non-immobilized antibodies were then removed from the capillary by washing with a buffer containing 0.05% Tween and 0.03% sodium azide in 1X PBS pH 7.4 at a flow rate of 50µL/min using a syringe pump. Each capillary was washed with 30µL of wash buffer in each step. Subsequently, a blocking buffer containing 2% FBS in 1X PBS pH 7.4 buffer was introduced into the capillaries for 1 hour at room temperature to block the vacant sites and prevent non-specific binding. MPO solutions at different concentrations or processed animal samples were then introduced into the pAb immobilized capillaries and allowed to interact with the pAb for
one hour at room temperature followed by injection of the wash buffer 1X PBS pH 7.4 containing 0.01% tween. QD conjugated mAb (QD-Ab) at 100nM concentration was then introduced into the pCAP, incubated at room temperature for one hour followed by washing with the wash buffer 1X PBS pH 7.4 containing 0.05% Tween. The fluorescence intensity of the QD-Ab from the capillaries was captured either using a fluorescent microscope or a monochrome CCD camera.

Several capillaries were imaged to evaluate and optimize the immobilization and reaction conditions by fluorescence microscopy (Leica DMRX upright fluorescence microscope) or CCD camera. Calibration curves were generated using known dilutions of MPO and this allowed us to determine the lowest limit of detection (LOD) of the whole assay. The amount of fluid inside the capillary was restricted to 1-2 µL by limiting the length of the capillary. The assay was then carried out to determine its sensitivity and selectivity for detecting MPO in solution and in animal stool samples. Figure 13 illustrates the entire protocol of the assay and Figure 14 summarizes the steps of the sandwich assay followed.
Figure 13: Steps of the assay for Myeloperoxidase Detection
Figure 14: Schematics of the Antibody Immobilization and Sandwich Assay inside the capillary set up
4.2.3. Optical Detection Set Up

In this project, three different optical detection set ups were evaluated. Two out of these three configurations provided useful results and could be used for the assay. During the development of the optical set up, LEDs with two different optical outputs were used. One LED had a 10mW optical output whereas the other had a 300mW optical output. The 10mW LED will be referred to as “Low Power LED (LPLED)” and the 300Mw LED will be referred to as “High Power LED (HPLED)” in the rest of this thesis. The three optical configurations evaluated were:

1. Waveguide Illumination Mode
2. Multiple UV-LED Array
3. Side-Illumination Mode

4.2.3.1 Waveguide Illumination Mode

In this configuration, each PMMA capillary (pCAPs) was inserted in a larger glass capillary for experiments where the capillary will act as waveguides. Excitation of QDs was achieved by coupling the UV light through the ends of the capillary; light propagates through the walls of the capillary tube exciting the QDs within the evanescent field. Figure 15 shows the schematics of this configuration, which was tested using both Low Power (LPLED) and High Power (HPLED) LEDs.
In the case of LPLED, a three axis manual micromanipulator was used to align the capillary along the axis of the LED to ensure maximum coupling of light for QD excitation. In this case a long pass filter was used to cut the excessive UV light and a cylindrical mirror was used to collect the scattered fluorescent light. The images for various concentrations of QD dilutions were captured using the COHU 4900 camera.

Figure 15: Schematic Illustration of Waveguide Illumination Mode

In the case of HPLED, the UV LED was aligned using a three axis micromanipulator to couple the light into the capillary. In this case, no cylindrical mirror was used to collect the scattered light. The images for various concentrations of QD dilutions were captured using the Sting Ray Camera.
4.2.3.2 Side Illumination Mode

This illumination mode was employed using just the High Power LED. This illumination mode was employed for High Power LED because the key components which were
identified to improve the signal to noise ratio included use of high power LED, Side Illumination mode and a better camera (Sting Ray Camera). An aspheric lens with a focal length of 6 mm (f=6mm), was used for focusing the UV light to a spot size of ~1.5 mm while a separator mounted in front of the lens holder allowed us to position the capillary in the focal plane of the lens. A three axis manual micromanipulator was used to align the UV. The capillaries were imaged with a CCD camera positioned at angle 90° to the UV source. The schematics of this configuration are shown in the figure below.
Figures 17-19 shows the configuration for the Side Illumination mode. In this mode, first capillaries are preassembled with stoppers (shown in figure 19) at predetermined distances. While taking the reading the capillaries are slid into the sample holder by compressing the spring loaded arms of the sample holder. Once they are placed properly in the sample holder, the arms of the sample holder are released to hold the capillary straight and in place for taking the fluorescence reading Tabs on the sample holder ensure that the capillary when placed properly remains at the focal plane of the focusing lens.

Figure 18: High Power LED Side Illumination Mode
Figure 19: Sample holder and Capillary with stoppers attached
4.3 Preparation of Animal Stool Samples

Stool samples from animals fed with Dextran Sodium Sulfate to simulate human Irritable Bowel Disease (IBD) were collected and frozen immediately. Myeloperoxidase (MPO) present in such stool samples simulating human was extracted by following the protocol previously established by Lettesjo et al.\textsuperscript{74}, using a special extraction buffer.

4.3.1 Extraction Buffer Preparation

The extraction buffer was prepared in such a way that it contains 12mM EDTA, 1% Fetal Bovine Serum (FBS), Protease Inhibitor (consisting of AEBSF 0.2 mM, E-64 1.4 µM, Bestatin 13 µM, Leupeptin 0.09 µM, Aprotinin 0.03 µM, EDTA 0.1 mM), 20% Glycerol and 0.05% Tween 20. When not in use the extraction buffer should be stored at 4\textdegree C.

4.3.2 Stool Sample Preparation Protocol

The stool samples were weighed and diluted by 5 times (vol/wt) by adding extraction buffer. The mixture was kept at 4\textdegree C for 15 minutes to hydrate the stool sample before homogenization. The hydration step was followed by sample homogenization (Tissue-Tearor Model No.: 985-370, Type 2, Biospec Products Inc) at 5000 RPM till a stable suspension could be obtained. Typical time required for homogenization varied between 15-60 seconds. The homogenized sample was allowed to mix with extraction buffer for 15 minutes at 4\textdegree C, followed by centrifugation at 14000 RPM at 4\textdegree C for 30 minutes. The
supernatant was collected without disturbing the pellet and it was stored at -20°C till further use.
5. RESULTS

5.1 Primary Antibody Immobilization

Immobilization of the antibody is an important parameter for the performance of a biosensor. The amount of antibody that is immobilized on the surface of the biosensor determines the performance of the antibody. Before performing any assay inside a capillary set up, it was important for us to determine whether the antibody is successfully immobilized on the inner walls of the capillary or not and at what concentration we reach saturation. In order to achieve this, the pCaps were functionalized with 1N NaOH for 1 hour and then activated with EDC/NHS solution for 5 hours (see Methods section for more details). Once the capillaries were activated, they were incubated with various concentration of QD-Ab conjugate for 16 hours at 4°C. The images were captured using the Leica Fluorescent Microscope at 10X magnification, 840ms frame speed and 12.2 gain. The frame speed and gain were chosen such that the CCD camera of the microscope saturates when capillaries were filled with just 100Nm QD-Ab solution.

In this experiment, the activated capillaries were incubated with 10, 25, 50, 100 and 350 nM of QD-Ab conjugate in triplicates at 4°C for 16 hours.

The images captured were analyzed using Image J. Briefly, raw images were cropped using Photoshop and a fixed size of images was used for measuring the average intensity. Figure 20 shows the schematic of image analysis that was used.
Figure 20: Steps in analysis of microscopic images
The results from Figure 20 show that we achieve maximum antibody coverage when the pCaps are exposed to 100nM or higher concentration of antibody. The results from this experiment were used in future experiments for carrying out the assay, i.e., for primary antibody immobilization, the capillaries were incubated with 100nM of primary antibody solution.

Figure 21: Graph shows the binding of various concentration of QD-Ab on the treated pCap surface

\[ R^2 = 0.95153 \]
Table 2 shows the intensity values for different concentration of QD-Ab immobilized on the surface of the capillary.

<table>
<thead>
<tr>
<th>QD-Ab Concentration (nM)</th>
<th>Average Intensity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>350</td>
<td>31.78</td>
<td>0.98</td>
</tr>
<tr>
<td>100</td>
<td>29.24</td>
<td>2.52</td>
</tr>
<tr>
<td>50</td>
<td>27.13</td>
<td>3.03</td>
</tr>
<tr>
<td>25</td>
<td>24.94</td>
<td>2.8</td>
</tr>
<tr>
<td>10</td>
<td>20.03</td>
<td>1.63</td>
</tr>
</tbody>
</table>

**Table 2**: Intensity values of capillaries immobilized with various concentrations of QD-Ab

Table 2 shows the intensity values for different concentration of QD-Ab immobilized on the surface of the capillary.
5.3. MPO Assay - Proof of Concept

After successfully immobilizing the antibody on the surface of the capillary, it was important for us to show that the whole assay can be performed inside the pCaps reliably. In order to achieve this, we started with an experiment at higher concentrations of MPO. The whole idea behind performing this experiment was to show that the sandwich assay can be performed inside the capillaries.

In this experiment, along with the assay performance, the effect of blocking was also evaluated. Two set of pCaps were immobilized with 100Nm primary antibody and one set of capillary were incubated with 1X PBS ph 7.4 buffer containing 2% Fetal Bovine Serum (FBS). For each set, MPO assay was carried out in triplicates.

The concentrations of MPO used for this experiment were 10, 100, 250 and 500nM. After, MPO capture step the pCaps were incubated with the 500nM QD-Ab. Since, this was experiment was done to check whether the assay works or not, we used 500nM QD-Ab. Except for this experiment, 100nM QD-Ab was used for all other experiments. The images were analyzed using Image J in the same way as mentioned in the previous section.

The results show that the assay worked for both pCaps sets, i.e. with and without FBS blocking. However, for pCaps without blocking higher intensity values were obtained. The pCaps incubated with blocking agent demonstrated lower (lower by almost 30-50%) fluorescence intensity values.
Figure 22: Graph showing the intensity values for various concentration of MPO for capillaries with and without blocking
The difference in intensity values for capillary with and without blocking for various concentrations of MPO could have been observed due to the fact that the blocking agent occupies vacant space on the surface of capillary, thereby, reducing the chances of non-specific binding of MPO or QD-Ab on the vacant surface.

Figure 23 shows the effect of using a blocking buffer during assay. From the figure it is clear that the addition of blocking buffer reduces the non-specific binding of either MPO or QD-Ab on the walls of the capillary.

<table>
<thead>
<tr>
<th>MPO Concentration</th>
<th>Blocking</th>
<th>Standard Error</th>
<th>No Blocking</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>130.80</td>
<td>10.33</td>
<td>243.69</td>
<td>6.18</td>
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<tr>
<td>250</td>
<td>77.87</td>
<td>11.19</td>
<td>125.35</td>
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<tr>
<td>100</td>
<td>54.30</td>
<td>6.34</td>
<td>93.75</td>
<td>19.30</td>
</tr>
<tr>
<td>50</td>
<td>41.48</td>
<td>6.90</td>
<td>88.01</td>
<td>9.10</td>
</tr>
<tr>
<td>10</td>
<td>34.89</td>
<td>5.74</td>
<td>35.46</td>
<td>3.56</td>
</tr>
<tr>
<td>0</td>
<td>19.58</td>
<td>1.33</td>
<td>18.01</td>
<td>1.98</td>
</tr>
</tbody>
</table>

Table 3: Intensity values of capillary with and without blocking for various concentrations of MPO
Capillary with 500nM MPO and Blocking Buffer

Capillary with 500nM MPO and No Blocking Buffer

Figure 23: Comparison of effect on blocking on capillaries with 500nM MPO
5.4 Low Power LED Optical Set Up Development

Demonstration of the assay performance as an MPO sensor using the microscope gave us enough confidence to develop an optical configuration where we could detect fluorescence at lower concentrations of QDs. For initial development of the appropriate optics, different concentrations of QD solutions were used in the pCaps instead of performing the whole conjugation assay. We evaluated two configurations with the Low Power LED: the waveguide illumination mode and the integrated waveguide mode using multiple LEDs. For the development of the LP LED optical set up the COHU 4900 CCD camera was used.

5.4.1. Wave Guide Illumination Mode

The basic geometry of this particular set up is shown in the materials and methods section. In this set up, a spherical mirror was used to reflect the scattering fluorescent light from the capillaries towards the detector. Figure 23 illustrates the set up with a spherical mirror. When a PMMA capillary filled QDs is excited, the fluorescent light travels in all 360° directions. This happens because of the cylindrical geometry of the capillary. In order to gather the maximum possible light from the capillaries, a spherical mirror was employed. The capillaries are placed in the focal plane of the mirror such that the maximum amount of light gets reflected back towards the detector.
In order to evaluate this set up, QD solutions at concentrations of 0.5, 1, 2.5, 5 and 10nM were used. For each concentration, the data was gathered in triplicate.

The images were captured using COHU 4900 camera with shutter speed at 8ms. The average intensity for each image captured by the camera was calculated using a Matlab...
Code. An image size of 300 x 200 pixels was taken for analysis. Graph in Figure 25 demonstrates a linear relationship between the intensity at various QD concentrations.

<table>
<thead>
<tr>
<th>QD Concentration</th>
<th>Average Intensity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>49.29</td>
<td>0.70</td>
</tr>
<tr>
<td>5</td>
<td>39.40</td>
<td>0.25</td>
</tr>
<tr>
<td>2.5</td>
<td>36.62</td>
<td>0.36</td>
</tr>
<tr>
<td>1</td>
<td>33.57</td>
<td>0.82</td>
</tr>
<tr>
<td>0.5</td>
<td>29.94</td>
<td>2.67</td>
</tr>
<tr>
<td>0</td>
<td>27.78</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Table 4: Intensity value for various concentrations of QD solutions
The results show a linear relationship between fluorescence intensity and QD concentration with a correlation coefficient $R^2$ value of 0.92. From the results of this experiment it can be concluded that this optical configuration is adequate in quantifying low concentrations of QDs.

5.4.2 Multiple LED Integrated Waveguide Mode

Once the waveguide illumination mode was established, the next goal was to increase the fluorescence signal from the capillaries by increasing the power of the excitation source. In order to achieve this, an array of 15 Low Power LEDs was used to excite the QDs in the capillary and the fluorescence signal was collected along the length of the capillary. Figure 25 shows the schematic of this set up.

Figure 25: Schematic of multiple LED integrated waveguide mode.

Figure 26: Figure showing multiple LED array set up. In this set up the signal was collected at the end of the capillary unlike the previous set up.
To test this set up for one specific concentration of QD solution, one set of capillaries were filled with 2.5nM QD solution and the second set of capillaries were filled with PBS pH 7.4 Buffer. The images were captured using the COHU camera and the average intensity value for each capillary was calculated.

![Figure 27: Comparison of intensities for capillaries filled with buffer and 1nM QD solution](image)

The results (Figure 27) show that there is hardly any difference between the intensity values of blank and capillaries loaded with 2.5nM QD solutions. This set up was not tested further for other QD dilutions because the ratio between the intensity of blank and 2.5Nm QD solution was 1.02, whereas the ratio between the intensity of value of the
waveguide mode was 1.32. In this set up, only a circular 6 x 6 pixel size window could be used for analysis. This made the analysis part really difficult.

<table>
<thead>
<tr>
<th>QD Concentration (nM)</th>
<th>Intensity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>236.1</td>
<td>0.55</td>
</tr>
<tr>
<td>1nM</td>
<td>241.7</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Table 5: Intensity value for 2.5Nm QD and capillaries filled with PBS Buffer
5.5 Low Power LED MPO Assay

The goal of this experiment was to perform the sandwich assay and determine the lowest detectable level of MPO inside the capillary using the successful waveguide illumination mode.

In this experiment, 0.1, 0.5, 1, 2.5, 5 and 10nM concentrations of MPO solutions were used to test the sensitivity of the sensor. The assay was performed in duplicates for each concentration and for each test 5 capillaries were used. The images were captured using the COHU 4900 CCD camera with a shutter speed of 16ms and the average intensity of each image for a window size of 300x200 pixels was calculated.

<table>
<thead>
<tr>
<th>MPO Concentration (nM)</th>
<th>Intensity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>85.46</td>
<td>1.46</td>
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<tr>
<td>5.0</td>
<td>76.01</td>
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<td>1.04</td>
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<tr>
<td>0.1</td>
<td>55.12</td>
<td>0.92</td>
</tr>
<tr>
<td>0.0</td>
<td>45.76</td>
<td>1.66</td>
</tr>
</tbody>
</table>

Table 6: Intensity value for various concentrations of MPO obtained using waveguide illumination mode.
Figure 28 demonstrates a non-linear relationship between the concentrations of MPO in solution and the fluorescence intensity obtained using the capillary in a waveguide mode with LPLED as the excitation light source. Detection down to 0.1Nm (100 pico molar) is possible; however, the resolution of the system decreases as the concentration of MPO decreases.

Figure 28: The graph shows the sensitivity of the sensor towards MPO at various concentrations.
We believe that the loss in resolution at lower concentrations stems mostly from limitations in the optical hardware i.e. our CCD camera, an analog camera with maximum integration time (shutter speed) of 16 ms. In addition; the low power of LED is another factor which affects the level of excitation of the QDs. Furthermore, perfect alignment of the capillary on the focal plane of the mirror and the proper coupling of light from excitation source is extremely difficult.

In order to overcome the limitations of the LPLED set up, a high power LED was used and different excitation modes were tested.
5.6 Development of the High Power LED Optical Configuration

The goal of this experiment was to establish an optimal optical detection set up which works for HPLED. For the HPLED optical set up development two modes of illumination were tested: the Waveguide mode and the side illumination mode as described in the materials and methods section.

In this experiment 0.05, 0.1, 0.5 and 1nM concentrations of QD solutions were used for testing the efficacy of the two excitation modes. An aspherical lens was used to focus the LED light on the capillaries. The reason for using the lens was to increase the optical power incident on the capillaries. When the HPLED is used without a lens then its optical output is $\sim 80 \text{mW/cm}^2$ whereas in combination with a lens the output is $190 \text{ mW/cm}^2$. The optical output of the High Power LED was measured using UV Power Meter. In this experiment, the fluorescence intensity at various locations of the capillary with side illumination was collected by moving the excitation source that was mounted on a translation stage. This allowed us to collect at a rapid rate statistically reliable data from individual capillaries mounted on a custom made spring loaded sample holder, which kept the capillary stretched and aligned. Figure 29 shows the set up, the UV LED is mounted on to a 3 axis macro-manipulator. The UV LED is moved along the length of the capillary to excite the QDs inside the capillary.
Figure 30 shows the effect of mode of excitation on pCaps loaded with QD solutions.

Table 7: Intensity values for various concentrations of QD using two modes of excitation.

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Waveguide Mode</th>
<th>Std Error</th>
<th>Side Illumination Mode</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40.3</td>
<td>1.20</td>
<td>153.33</td>
<td>14.52</td>
</tr>
<tr>
<td>0.5</td>
<td>33.3</td>
<td>1.76</td>
<td>82.50</td>
<td>0.41</td>
</tr>
<tr>
<td>0.1</td>
<td>19.0</td>
<td>1.00</td>
<td>28.00</td>
<td>2.31</td>
</tr>
<tr>
<td>0.05</td>
<td>18.0</td>
<td>0.00</td>
<td>19.67</td>
<td>1.20</td>
</tr>
<tr>
<td>0</td>
<td>16.3</td>
<td>0.33</td>
<td>16.33</td>
<td>0.33</td>
</tr>
</tbody>
</table>
The advantages of side illumination can be clearly seen although the difference diminishes at concentrations below 100 picoMolar (0.1nM).

For the side illumination mode the fluorescent intensity was higher than with the waveguide illumination mode. The loss in fluorescence in the waveguide mode can be attributed to several factors: 1) optical misalignment in the waveguide mode leading to reduced light coupling efficiency, 2) insufficient path length resulting in decreased fluorescence volume and 3) most importantly due to the higher refractive index of the PMMA capillary compared to the refractive index of the buffer inside the capillary, hence, the PMMA capillary doesn’t work as a perfect waveguide for illuminating the QDs in the solution.

Figure 30: Shows the comparison of fluorescence intensities of QD solutions obtained with side illumination and waveguide modes. pCaps were inserted inside a glass capillary when imaging in the waveguide mode.
Side illumination on the other hand results in exciting almost all of the QDs in a volume of \(\sim 50\text{nL}\) (beam diameter = 1mm) and this could be the main reason for the observed difference in intensities between the two illumination modes at relatively higher concentrations (>100 pM). As the concentrations approach the 50pM range the difference in intensity is practically zero. Hence, for our purpose side illumination appears to be a better choice due to its simplicity and superior performance.

From this experiment onwards, side illumination mode was employed for all future experiments with HPLED (~190Mw/cm\(^2\)) as the excitation source.
5.7 Primary Antibody Circulation Experiment

As mentioned earlier, antibody immobilization is a critical component of a biosensor’s performance and it is affected by various factors. One such factor is the mixing of the antibody solution to provide a homogeneous and continuous supply of antibody and accelerate the immobilization process. When antibody is circulated through capillaries it can reach the surface of the capillary faster and adsorption on the polymer surface is accelerated resulting in faster immobilization rates. In this experiment, we explored the effect of a static immobilization process versus a dynamic flow and measured the effect on the rates of antibody immobilization. The main goal of the experiment was to reduce the time required for the antibody immobilization step. In order to achieve this, after initial capillary activation, for one set of capillaries the primary polyclonal antibody was circulated through the capillary using the peristaltic pump at 30µL/min. For the second set, the capillaries were filled with primary antibody solution and left at 4°C for 16 hours. Following antibody incubation, both sets of capillaries were incubated with 10nM MPO to check the effect of circulation on sensor performance.

The images were captured using the COHU 4900 camera and the images were analyzed using Image J to calculate the average fluorescence intensity values.

Figure 31 shows that all capillaries where the antibody was circulated for the immobilization step had higher intensity values compared to the capillaries where static immobilization was employed.
Table 8: Comparison of intensity values for capillaries with and without primary antibody circulation step.

<table>
<thead>
<tr>
<th></th>
<th>Average Intensity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulation</td>
<td>80.67</td>
<td>5.51</td>
</tr>
<tr>
<td>No Circulation</td>
<td>60.83</td>
<td>6.22</td>
</tr>
</tbody>
</table>

Figure 31: Effect of circulation on during antibody immobilization process
This result confirms that at least the antibody circulation at room temperature for one hour doesn’t affect the sensor performance adversely and hence, it can be employed in future experiments to bring down the capillary preparation time from 22 hours to merely 7 hours. Initially, the capillary activation step was for 5 hours and the antibody immobilization use to take 16 hours. Now the antibody immobilization step takes just one hour, hence, this reduced the capillary preparation time to 7 hours from 22 hours. In order to save time in capillary preparation in future experiments, the antibody solution was circulated through the capillary for one hour.
5.8 High Power LED MPO Assay

After successfully identifying an optical configuration and a satisfactory immobilization scheme, we proceeded to translate these improvements into a full MPO immunoassay at lower concentrations. From the previous results we gained enough confidence that the changes implemented can improve our signal to noise ratio at lower concentration of MPO, i.e, 100 pico molar. In order to further improve the signal to noise ratio we used, for all subsequent experiments the Stingray CCD camera which has with superior features compared to the COHU CCD camera.

MPO concentrations of 1, 0.5 and 0.1nM were prepared in duplicates (Total N = 9) and the assay was performed inside antibody immobilized capillary. Fluorescence intensity at various locations on a capillary was collected by moving the excitation source that was mounted on a translation stage. The images were captured at an integration time of 100 µs with maximum gain and shutter speed of the camera. The captured images were analyzed with Image J. A window size of 30x15 was use to calculate the average intensity between the two bands of the capillary.
Table 9: Intensity values for various concentrations of MPO for High Power LED set up
Figure 32 shows the improved performance of the sensor at lower concentrations of MPO, i.e., the ratio between the signals of blank and 0.1nM MPO is almost 1:2, which is much better than the waveguide mode set up employed in the case of LPLED, where the signal to noise ratio was 1:1.3.

Figure 33 shows the comparison of LPLED waveguide set up and HPLED side illumination set up. From the graph we can see a better signal to noise ratio in case of HPLED set up.

Figure 33: Comparison of LPLED side illumination with HPLED waveguide geometry: Intensity values are corrected by the control intensity
Figure 34 shows spot free images for lower concentrations of MPO. These images were captured at various locations on a capillary and demonstrate uniformity of the fluorescence signal. The bands that appear in the CCD images are result of the architecture of the optical set up and capillary geometry. The intensity from the bands of the capillary were not used in analysis because they represent the leakage of UV light through the band pass filter.
5.9 NHS vs Sulfo-NHS Treated Capillaries

The primary purpose of this experiment was to evaluate the effect of using sulfo-NHS versus using NHS along with EDC for antibody immobilization. The non-sulfonated NHS is less water soluble and hydrolyzes faster in water compared to the sulfonated-NHS. The main advantage of using sulfo-NHS in EDC reactions is to increase the stability of the active intermediate of EDC during the covalent bonding formation process with either amine or carboxyl group. In this experiment, we tried to explore the effect of sulfo-NHS use on sensor performance.

In this experiment, 30mM of EDC and NHS solutions were prepared in PBS pH 6.0 buffer. After NaOH functionalization of the capillaries, the capillaries were activated with EDC/Sulfo-NHS instead of EDC/NHS solution. The EDC/sulfo-NHS solution was circulated through the capillary for 1 hour at room temperature followed by circulation of 100nM primary antibody solution for 1 hour at room temperature. Once the capillaries were ready, the whole sandwich assay was carried out to see the effect of sulfo-NHS use on the sensor performance for detecting 0.1, 0.5 and 1nM MPO.

From Figure 35 it is clear that there is a good correlation of fluorescent intensity values between sulfo-NHS and NHS treated capillaries. This was further confirmed by performing a t-test on the intensity values at different concentration. The p value obtained from the t-test was ~0.95, which is greater than 0.05, hence, statistically there is no significant difference between the two groups.
Figure 35: Comparison of fluorescence intensity at various concentration of MPO for NHS and Sulfo-NHS treated capillaries
Hence, it can be concluded that the use of sulfo-NHS didn’t affect the performance of the sensor. However, the use of sulfo-NHS reduced the capillary preparation time from 7 hours to 3 hours. This reduction in time happened because in previous experiments EDC/NHS was circulated for 5 hours at room temperature however, EDC/Sulfo-NHS is circulated for 1 hour thereby reducing the capillary preparation time from 7 hours to 3 hours.

Table 10: Comparison of Intensity values for various concentration of MPO treated with NHS and Sulfo-NHS. P-value obtained after performing t-test gives the correlation between two groups.

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Intensity (Sulfo-NHS)</th>
<th>Std Error</th>
<th>Intensity (NHS)</th>
<th>Std Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>207.83</td>
<td>7.963458</td>
<td>201.94</td>
<td>7.18</td>
<td>0.95</td>
</tr>
<tr>
<td>0.5</td>
<td>126.75</td>
<td>4.597705</td>
<td>128.81</td>
<td>12.42</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>75.5</td>
<td>4.327721</td>
<td>81.08</td>
<td>5.51</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>36.75</td>
<td>3.037954</td>
<td>48.20</td>
<td>2.85</td>
<td></td>
</tr>
</tbody>
</table>
5.10 Assay Specificity Test

The purpose of the following experiment was to test the specificity of the sensor and the assay. To test the specificity, instead of MPO alone Human Matrix Metalloproteinase I (MMP 1) was used in the same experiment. Since the polyclonal antibody (capture molecule) and the monoclonal antibody (reporter molecule) will bind only to MPO, we shouldn’t be seeing any fluorescence from the capillaries infused with MMP 1.

The experiment was divided into two sets. In the first set of experiments, the immobilized capillaries were incubated with 150 ng/mL of MMP 1. In the second set of experiments, the capillaries were immobilized with a solution containing a mixture of 50:50 MMP 1 and MPO at a total concentration of 150 ng/mL each. The goal of the second experiment was to check whether the sensor can detect MPO in the presence of another biomolecule.

The results show that the sensor can detect successfully MPO in the presence of MMP 1. The capillaries which were incubated with MMP 1 alone had slightly higher intensity value than the negative control capillaries. This could be due to the non-specific binding of QD-Ab on the surface of capillary tube or a certain degree of non-specificity of the antibodies for MMP 1.

This experiment illustrates the specificity of the antigen/antibody reaction for this particular sandwich assay.
Table 11: Comparison of fluorescence intensity from capillaries with MMP and MMP+MPO containing 150ng/mL of MMP and MPO.

<table>
<thead>
<tr>
<th></th>
<th>Intensity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP</td>
<td>47.94</td>
<td>1.40</td>
</tr>
<tr>
<td>MMP - MPO</td>
<td>163.71</td>
<td>10.77</td>
</tr>
<tr>
<td>Control</td>
<td>34.71</td>
<td>2.12</td>
</tr>
</tbody>
</table>

Figure 36: Comparison of fluorescence intensity from capillaries with MMP and MMP+MPO containing 150ng/mL of MMP and MPO.
5.11 Spiked Animal Sample

The next step in validating the sensor and the assay was to test their performance in a more complex system simulating clinical samples. Presence of various biologically relevant moieties that are present in the animal stool samples is a major concern in developing a sandwich assay since the entire assay relies on the specificity and cross reactivity of the polyclonal (capture antibody) and the monoclonal antibody (reporter molecule) towards the antigen. Sensitivity of the QLISA protocol thus depends on the sensitivity and specificity towards one another in the pAb/MPO/QD-Ab sandwich. Although chemistry optimization steps were taken to minimize nonspecific interactions between the sensor substrate and the analyte, it is imperative that the robustness of the protocol and the device be evaluated with actual samples rather than solutions of the antigen.

The assay protocol was tested in spiked animal stool samples to evaluate its ability to detect MPO at trace levels in biological samples. Stool samples from disease-free mice were collected and prepared as described in the Materials and Methods section. The extract obtained served the purpose of being the control and external MPO was added to the extract (spiking). The stool sample extract was spiked such that the final concentration of MPO in the extract was 1, 0.5 and 0.1nM respectively. In order to get an extract with 1nM external MPO, 10 µL of external MPO (10 nM) was added to 90 µL of the stool extract and so on. In this mouse model (Karwa et al. 2007), before inducing disease, the animals bear no MPO in their stools. Intensity values of MPO obtained from
the spiked stool extracts were then compared with the intensities from MPO dilution experiment done previously.

Figure 37: Fluorescence intensity from spiked animal stool samples. Fluorescence intensity values obtained from spiked animals were compared with fluorescence intensities of MPO in solution.
Figure 37 shows the fluorescence intensity data from stool samples collected from the animals along with data from MPO in solution (standard curve). Spiked stool samples exhibit response that is similar to that of MPO in solution, illustrating the specificity of the antigen/antibody complex and the robustness of the assay protocol. The intensity value obtained from the stool sample that does not contain any MPO is essentially the same as the control capillaries of the MPO solution set, indicating the absence of non-specific interaction between the capture antibody and/or the mAb. This was further confirmed by the t-test results; with $p > 0.94$ indicate that there is statistically no difference between the two data sets.

<table>
<thead>
<tr>
<th>MPO Concentration (nM)</th>
<th>Spiked Animal Sample</th>
<th>Standard Error</th>
<th>Standard Curve</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>198.35</td>
<td>13.36</td>
<td>201.94</td>
<td>7.18</td>
<td>0.94</td>
</tr>
<tr>
<td>0.5</td>
<td>136.78</td>
<td>4.36</td>
<td>128.81</td>
<td>12.42</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>90.78</td>
<td>4.45</td>
<td>81.08</td>
<td>5.51</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>48.84</td>
<td>5.85</td>
<td>48.20</td>
<td>2.85</td>
<td></td>
</tr>
</tbody>
</table>

Table 12: Comparison between the intensities of Spiked Animal Data and MPO Standard Curve
5.12 Animal Samples

The spiked animal data were considered as an encouraging first step towards using this assay in a full disease model to quantify the presence of MPO in stools. The mouse model and the use of QDs to evaluate inflammation, established by Karwa et al.\textsuperscript{63} were used for this experiment.

Stool samples were collected on different days after induction of colonic inflammation in mice. For this experiment, stool samples collected before induction of inflammation (Day 0), on day 5 and on day 7 of inflammation were used for analysis. For Day 7, samples from 5 animals were used for analysis and for Day 0 and Day 5, samples from 3 animals were used. The assay run was repeated twice and each run was performed in triplicate.

Figure 38 shows the results of the assay for animal samples collected at different days. We can see a clear difference in the level of fluorescence intensity for Day 7 samples when compared with the Day 5 and Day 0 samples. This is observed due to the over expression of MPO in animals after they are fed with Dextran Sulfate Sodium (DSS) for 7 days. This result shows that the sensor and the assay protocol developed can differentiate between diseased animals with inflammation and healthy animals.
Figure 38: Comparison of Intensity values for stool samples collected on different days after induction of inflammation.

<table>
<thead>
<tr>
<th></th>
<th>Intensity</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>87.45</td>
<td>4.35</td>
</tr>
<tr>
<td>Day 5</td>
<td>49.61</td>
<td>1.09</td>
</tr>
<tr>
<td>Day 0</td>
<td>37.22</td>
<td>2.38</td>
</tr>
</tbody>
</table>

Table 13: Comparison of intensity for various samples collected on various days after induction of inflammation.
To further confirm the reliability of the assay and the sensor, ELISA was performed on the stool samples.

For an ELISA assay 100µL of sample is required, therefore we could only perform the ELISA assay on 4 samples from Day 7, one sample from Day 5 and 3 samples from Day 0. For this experiment, the Zen Myeloperoxidase Kit purchased from Invitrogen was used.

Figure 39 shows the comparison of in-house MPO assay and ELISA. For day 7 samples, ELISA detected ~7.5 ng/mL of MPO in prepared stool aliquots, whereas, the in-house MPO assay detected ~13ng/mL of MPO.

Figure 39: Comparison of detection of MPO by a commercial ELISA kit and QD-Assay for Day 7 diseased samples
From the results it can be concluded that a similar pattern was observed for both ELISA and in-house MPO assay. Even though the assay and the sensor developed in the lab showed 77% higher concentration of MPO than the commercial ELISA kit for Day 7 samples. However, when t-test was performed no statistically significant differences between the two results were observed. This shows that there is a fair correlation between the two results.

The higher value of MPO level in the sensor could have been detected due to several factors: 1) For each pair of antibody/antigen the affinity varies and the antibodies used in commercial ELISA and in-house assay were different and this would account for the observed differences. 2) Non-specific binding of QD-Ab to various biological molecules in the stool sample or to the walls of the capillary could have contributed to a higher level of MPO detection.

<table>
<thead>
<tr>
<th>Day 7 Sample</th>
<th>MPO (ng/mL)</th>
<th>Standard Error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD-Assay</td>
<td>12.67</td>
<td>1.92</td>
<td>0.17</td>
</tr>
<tr>
<td>ELISA</td>
<td>7.14</td>
<td>1.93</td>
<td></td>
</tr>
</tbody>
</table>

Table 14: Comparison of performance of in-house QD-Assay and ELISA for Day 7 samples. p value of 0.17 (p>0.05) shows that there is no statistically significant difference between the commercial ELISA kit results and in-house QD-Assay.
6. Discussion

The unique optical properties of QDs such as broad excitation spectra, narrow emission spectra, large Stokes Shift, high resistance to photobleaching, higher photo-stability and stronger fluorescence makes them one of the most attractive fluorescent reporter molecules for a wide range of biological applications including biosensing and assay development applications\textsuperscript{76}. However, the higher cost of QDs has limited their application in biology. One such application is the development of assays. Usually, bioassays are carried out in 96 well plates which require at least 50 - 100µL of sample and reagents. The most common method followed for plate based assays is Enzyme Linked Immunosorbent Assay (ELISA). In order to capitalize on the superior properties of QDs we devised a capillary based immunoassay utilizing just 1-2µL of sample and reagents for detection of biomarkers thereby reducing the cost of reagents in the whole process. Also, a free standing capillary tube offers superior simplicity in manufacturing and handling compared to developing a full scale lab-on-a-chip type device. In order to test and demonstrate whether a capillary based sandwich immunoassay and the use of QDs could be integrated to detect an antigen, we targeted a ubiquitous inflammation marker Myeloperoxidase (MPO). We demonstrated the detection of MPO with QDs as reporter molecules inside the capillaries by using a fluorescent microscope. Even though very high concentration of MPO was used to demonstrate this, still it gave enough confidence to develop an optical set for detection of fluorescence from the capillary. The effect of using an optimal concentration of blocking agent was explored. Regions of
higher QD intensity in the absence of blocking agent were observed because after antibody immobilization few vacant sites remain which contribute to non-specific binding of QD-Ab. In order to overcome this, Fetal bovine serum (FBS) was added to the buffer which binds non-specifically to the vacant sites on the inner surface of the pCap. This experiment suggested that the QD based assay can be used to detect MPO.

In the next experiment the optimal optical set up was explored for detection of fluorescence from capillaries containing MPO. Two set ups i.e., waveguide mode and multiple array mode excitation were explored and it was found that the waveguide mode was better because of significantly reduced noise compared to the multiple LED array. The LED array was expected to give better results than the waveguide illumination mode; however, this failure can be attributed to the destructive interaction of photons from each LED there by leading to reduced excitation power. After the development of the optical configuration, the lowest limit of detection of MPO was explored using the waveguide illumination mode. A non-linear relationship was obtained for various concentrations of MPO. Even though the lowest limit of detection was found to be 0.1nM, the signal to noise ratio deteriorated rapidly for concentrations below 1nM MPO. This loss in signal intensity could be attributed to the limitation of the CCD camera (maximum 16ms shutter speed) and also to the alignment of the capillary. The capillary should always be aligned properly on the focal plane of the spherical mirror in use and also, it should be aligned along the UV excitation source for maximum coupling of light.
In order to overcome these challenges, a new high power LED was used along with an
apsherial lens to establish a side-illumination mode of excitation. This mode of
excitation gave superior results and it addressed the issue of complex alignment of the
capillary along the axis of the light source for maximum coupling of light into the
capillary. In this case, the alignment was very simple and straightforward and only a
small portion of capillary is excited by the light which results in excitation of almost all
QDs in that particular region leading to higher intensity value.

In the next experiment, continuous flow / mixing of the antibody was explored to
evaluate its effect on primary antibody immobilization. It was found that in the case of
continuous flow immobilization higher fluorescent intensities were observed. Mixing of
antibody molecules resulted in their rapid movement towards the surface of the
substrate and facilitated faster immobilization on the polymer surface. In the next two
experiments, a standard curve for the MPO assay at lower concentrations was established.
It was observed that for High Power side-illumination mode the signal to noise ratio
better and for 100 picomolar MPO was higher by 350% when compared with the Low
Power LED Waveguide Mode results. This was due to higher power of excitation beam
incident on the capillary.

In addition to sensitivity, specificity is the most important aspect of any assay
development or immunosensor development process. Given the multitude of things
happening in a human body it is important to determine the specificity of the
antigen/antibody reaction of the system. In order to test the specificity of the developed
assay, we tested its performance in the presence of another biomolecule MMP-1. The sensor was able to detect MPO in the presence of MMP 1 without giving any false positive result. This was followed up by a more complex system. External MPO at various concentrations were added to stool samples from control animals. The spiked stool samples exhibited response similar to that of MPO in solution, further illustrating the specificity of the antigen/antibody complex and the robustness of the assay protocol.

In the next experiment, stool samples from healthy and diseased animals (with inflammation due to simulated colitis) were used for checking the performance of the quantum dot based micro-capillary assay. By taking advantage of QDs we were able to detect the MPO in just one micro liter of sample from diseased animals. The results obtained from our experiments were compared with those of a commercial ELISA kit for MPO. The ELISA results further corroborated the performance of the QD assay developed for MPO detection. t-Test further confirmed that there is no statistically significant difference between the two assay results for Day 7 samples.

One of the major hurdles which our micro capillary assay faces could be the limit of detection. Even though our assay is able to differentiate between diseased and healthy samples, from a diagnostic point of view we would like to be able to monitor disease progression. With the current set up and the current limit of detection this cannot be achieved with the desired sensitivity. In order to achieve this, various parameters such as superior antibody immobilization process, increase in sample volume (from 1 to 5
microliters) and a superior optical set up are some areas where improvements can be made to enhance the performance of the QD capillary immunoassay.
7. Conclusion

A PMMA polymer capillary based assay has been developed which is capable of detecting analytes at picomolar level in micro liter volumes. The capillary immunosensor and the QD assay was developed and tested with Myeloperoxidase, a marker that is over expressed in the case of inflammation. The sandwich assay for MPO was first validated using a microscope. After this validation, different optical set ups were tested to establish the best performance of the system. Primarily, two different geometries, i.e., waveguide illumination mode and side illumination mode were used to excite the QDs inside the capillary either by using a high power LED or low power LED. The lowest limit of detection which could be achieved with this platform was approximately 100 picomolar (~15ng/mL) of MPO in solution for both High Power and Low Power LED. However, High Power LED side illumination mode performed better in terms of signal to noise ratio at lower concentrations. The side illumination mode also eliminated the problems pertaining to optical alignment. The results obtained indicate that the QD assay can be effectively used inside a microcapillary to detect analytes in biological samples such as stool samples and is capable of differentiating the diseased animals from the healthy ones. The result of the capillary based QD assay correlated well with commercial ELISA kit for determining the level of MPO in diseased animal stools samples. Hence, it can be concluded that the advantages of capillary based assay and superior photo-physical properties of QDs were integrated successfully developed and tested with MPO. The
main advantages are small sample volume (~1-2 μL) and reagent volumes (~10 μL) and inexpensive optics.
8. Future Work

In this research work, we successfully developed a Quantum Dot Based Microcapillary Imunosensor for detection of MPO, thereby laying foundation for future developments. There are various aspects on which improvements can be made in order to make the immunosensor more sensitive and high throughput.

In order to improve the sensitivity of the immunosensor various things can be employed. First and foremost, the amount of antibody immobilization on the surface of the antibody can be increased by using a non-zero crosslinker to enhance the sensor performance. Bai et al reported a study where they compared the effect of using a non-zero cross linker poly(ethyleneimine) PEI with a zero length cross linker Hexamethylene Diamine (HMD) on antibody immobilization on the surface of PMMA and its subsequent effect on the performance of the sensor\textsuperscript{75}. They reported that the PEI greatly improved the sensor performance mainly due to the spacer effect which it provided for antibody immobilization. Hence, in future by using non-zero length crosslinker we can increase our immunosensor performance.

One more way of increasing the sensor performance by using a different Quantum Dot probe, for e.g., streptavidin conjugated QD and biotinlyated secondary antibody. A study reported by the Zajac et al showed that the use of biotinlyated antibody and streptavidin QDs instead of QD-Ab conjugate increased their sensor performance level significantly\textsuperscript{59}. They reported detection in picograms/mL range (femtomolar region). Hence, in future this can be employed to check whether the sensor performance increases or not.
However, the main disadvantage of this method is the extremely high cost of the reagents.

One more way of improving the sensor performance is by using expensive optical set up hardware’s like a Laser excitation source and sensitive Photo Multiplier Tubes. However, use of these equipments will considerably increase the cost of the optical set up.

In the current set up, we are handling only one capillary at a time. Due to this the data acquisition process is very slow and labor intensive. In order to make this system high throughput, a multiple capillary holder set up can be devised in future to carry out the assay thereby making it fast, portable and automated.

All the above ideas can be implemented in future to improve the performance of the current QD based microcapillary immunosensor.
9. References

62. Flanigan SMaA. In Vivo Models of Inflammation: Animal Models of Inflammatory Bowel Disease.