Abstract

Towards a Microfluidics-Based Nucleic Acid Biosensor Using Immobilized Quantum Dot – DNA Conjugates for FRET Detection of Target Oligonucleotides

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The potential for an electrokinetically driven, FRET-based microfluidic biosensor for SNP discrimination has been explored. The method functionalized the glass wall of a PDMS/glass microfluidics channel with multidentate thiol ligands to noncovalently immobilize MPA capped CdSe/ZnS QDs. Single stranded probe DNA could then be immobilized to QDs and target material could be delivered electrokinetically to the sensing surface. SNP discrimination could then occur by manipulation of shear, electrical and thermal effects derived from the applied voltage. The stability of immobilized QDs was investigated by EOF experiments that applied 500 V and 100 V voltages for 10 minutes to initiate electrokinetic flow. Fluorescence intensity measurements showed nearly complete removal of QDs from slides when compared with controls at both voltages. Pressure driven flow experiments demonstrated reduced dissociation of immobilized of QDs compared to channels exposed to EOF. A covalent approach is likely necessary to ensure stability of immobilized QDs during EOF.
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List of Abbreviations

SNP - Single Nucleotide Polymorphisms
mRNA - Messenger Ribonucleic Acid
miRNA – Micro Ribonucleic Acid
DNA – Deoxyribonucleic Acid
RNA – Ribonucleic Acid
IC – Internal Conversion
ISC – Intersystem Crossing
FRET - Förster Resonance Energy Transfer
PCR – Polymerase Chain Reaction
nt – nucleotides
bp – base pair
EOF – Electroosmotic Flow
PNA – Peptide Nucleic Acid
FET – Field Effect Transistors
SWNT – Single Walled Nanotubes
NTFET – Carbon Nanotube Field Effect Transistors
QCM – Quartz Crystal Microbalance
RT-PCR – Real Time Polymerase Chain Reaction
SPR – Surface Plasmon Resonance
SP – Surface Plasmon
LOD – Limit of Detection
QD – Quantum Dot

APTMS – 3 – Aminopropyltrimethoxysilane

GOPS – 3 – glycidoxypropylsilane

MPA – 3-mercaptopropionic Acid

MAA – Mercaptoacetic Acid

DCC – N, N’ - Dicyclohexylcarbodiimide

DMAP – 4 – Dimethylaminopyridine

SDS – Sodium Dodecyl Sulfate

TCEP – (Tris(2-carboxyethyl)phosphine)

BTA – 1,3,5 benzentricarboxylic acid
Introduction

Nucleic acid sensors have become increasingly prominent in the literature because of the need for reliable technology in diagnostic medicine, veterinary medicine and the food and agriculture industries. These technologies take advantage of the ubiquity of nucleic acid target material in eukaryotes, prokaryotes and viruses and the inherent selectivity of Watson-Crick base pairing between complementary single stranded nucleic acids. Deoxyribonucleic acid (DNA) is a nucleic acid bio-polymer that serves as the genetic template for all prokaryotic and eukaryotic life on earth. The information it encodes is read as a linear string utilizing a four-letter alphabet. The monomers of these polymers are nucleotide bases and consist of a phosphate group, a deoxyribose sugar and one of four nitrogenous bases. Arrangement of the bases Cytosine (C), Thymine (T), Adenine (A) and Guanine (G) in a linear sequence can produce a unique genome that will code for the protein and peptide content of an organism. Bases are linked in linear sequence by a phosphodiester bond created by dehydration between two adjacent phosphate residues. Even though there are a large proportion of conserved sequences between different organisms, there are sequences that are exclusive to a particular species. Tools that can be used to identify conserved sequences include Genbank and BLAST, which are libraries of known nucleotide sequences. Unique sequences can be selected to construct probes, and these form the basis for the detection of certain target DNA through hybridization. The overwhelming majority of development of these nucleic acid sensors has centered on microarrays and biosensors where target is introduced to probe sequences immobilized onto a solid support.
Microarrays are manufactured by spotting tens to thousands of 10 – 100 µm sized zones of unique probe sequences of DNA/RNA material onto glass, silica or even plastic substrates\textsuperscript{6-8}. The major application of microarrays has been to observe the pattern of gene expression of thousands of gene transcripts at once in laboratory flora and fauna\textsuperscript{8-10} and has been used in genomic screening such as in study of delete human cancers\textsuperscript{6, 11, 12}. This approach has been invaluable in determining how the expression profile of one gene affects the transcription profile in other genes related to its function. Despite the advantages of the microarray approach there are drawbacks. They are expensive to manufacture, they do not show a great deal of reusability and sometimes the experiment is only concerned with a small number of genes or gene products in a sample\textsuperscript{8}.

These concerns represent opportunity for biosensors - devices that immobilize probe material directly to a transduction element that translates the bio-recognition event into a measurable analytical signal\textsuperscript{1}. Observing multiple genes concurrently requires long equilibration times to minimize non-specific binding events, multiple washing steps and complicated, time-consuming data analysis\textsuperscript{13}. Biosensors can significantly reduce the time for complete hybridization from typically several hours for microarrays to several minutes by limiting the number of genes being examined\textsuperscript{14}. The need for rapid screening is critical for improving point of care or high throughput applications. To illustrate, consider a patient infected with bacterial meningitis - a disease which can become life threatening quickly if treatment is not provided in a timely fashion\textsuperscript{15}. Accelerating the time required to make a positive diagnosis can drastically improve the patient’s chances for survival and reduce the probability of exposing the patient to the wrong medication.
This exposure can have detrimental side effects on the patient that can significantly squander health-care resources 15.

In addition to speed, the biosensor should provide high confidence that the signal generated from the annealing of target with probe sequences is due to fully complementary material and not to any non-selective adsorption. Compounding this issue are single nucleotide polymorphisms (SNP), which differ from the fully complementary conjugates by one base pair. The device therefore must exhibit stringently controlled selectivity and high sensitivity to distinguish between these sequences.

Developing a reusable sensor that can confidently detect and quantify the presence of a specific target in a period of minutes summarizes the requirements expected of most practical biosensors. The work done for this thesis explored the feasibility of development of a biosensing system for oligonucleotides that combines a novel method of fluorescence transduction, with microfluidic technology for sample handling and stringency control. The biosensing system involves immobilization of quantum dots that are functionalized with nucleic acid probes onto a glass/PDMS microfluidic channel wall, and delivery of fluorescent dye-labeled oligonucleotide targets to the sensing surface by electrokinetic flow. FRET between excited QDs and fluorescent dye serves as the transduction method. Electrokinetic microfluidic platforms enable precise control and delivery of target material to the sensing surface and can provide selectivity control and sensitivity enhancement 14, 16.

**Nucleic Acid Hybridization**

The formation of an energetically stable duplex of DNA is highly dependent on complementary pairing. Hydrogen bonding between nitrogenous bases on opposite strands
serves to hold the individual strands together. This interaction is specific in that A binds to T and G binds to C with the highest stability. The bases A and G are called purine bases, and contain two fused nitrogenous rings. C and T are pyrimidine bases that have a single nitrogenous ring. Standard Watson-Crick base pairing ensures that only three nitrogenous rings will be present per rung of the DNA ladder. In addition to hydrogen bonding, there are a number of other energy considerations in DNA hybridization. The hydrophobic nitrogenous bases are planar to the other bases in the DNA creating overlap between the pi orbitals of the conjugated ring systems referred to as pi ring stacking. Hybridization also causes dipole moments present on the phosphodiester backbone to point inwards towards the center of the duplex. Finally, before the strands come together there are a number of water molecules solvating the nitrogenous bases. When hybridization occurs the interior becomes hydrophobic causing these water molecules to be liberated to the rest of solution producing a net entropic gain with duplex formation.

Part of the energetic stability of duplex formation is also related to how many base mismatches occur along a sequence of a certain length. Increasing the length of the strands decreases the energy penalty associated with a mismatch. The thermal denaturation temperature (T_m) is typically used to distinguish between similar and fully complementary targets. The T_m can be defined as the temperature at which half the total double-stranded DNA has dissociated into single strands and it serves as one relative measure of thermodynamic stability. Spectroscopic determination of the T_m typically involves measuring the change in fluorescence or UV-absorption of the heterocyclic bases of DNA. Temperature change alters the ratio of double stranded to single stranded DNA, which affects the fluorescence and absorption efficiency of the nucleotide bases. Studies by Piunno and coworkers have demonstrated differences of 8 – 11 °C in the thermal
denaturation temperature of fully complementary and SNP targets in low stringency conditions with immobilized probe lengths of 20 nucleotides \textsuperscript{18}. Part of the contribution to the range of the change of melt temperature is whether the mismatch occurs at the center of a duplex or at the end. Mismatches at the center have been shown to destabilize hybridization to a greater extent than mismatches at the end of the duplex creating a greater temperature difference between SNPs in that event \textsuperscript{19}. By strictly controlling the variables that effect hybridization such as temperature, pH and ionic strength; differences in T\textsubscript{m} values between SNPs and fully complementary targets can be maximized and a greater selectivity imposed. Typical probe lengths used for biosensors vary between 10 and 30 bases since any mismatches will destabilize the duplex to a greater degree than if the strands were longer \textsuperscript{18}. Additionally, smaller probe lengths also show faster hybridization kinetics than longer probes, resulting in opportunity for higher throughput analyses \textsuperscript{20}.

**DNA analogs**

In addition to DNA, ribonucleic acids (RNA) are also utilized in biosensor design, albeit to a lesser extent. Messenger RNA (mRNA) is transcribed from coding regions of DNA and serves as a template for the production of a protein. Messenger RNA can be present in eukaryote cells in ~ 1000 fold excess of DNA providing a natural signal amplification if the mRNA is chosen as the target. Precautions in this experimental design are necessary to prevent target degradation as RNA hydrolysis enzymes are ubiquitous \textsuperscript{1, 20}. The presence of mRNA therefore indicates that viable cells are present in the sample whereas DNA cannot provide this information because of its longevity. Secondly, as
mentioned previously copies of RNA are present in large excess compared to copies of DNA, therefore lesser numbers of cells are required to develop an analytical signal.

Besides mRNA, there are several other types of RNA. Transfer RNA (tRNA) and ribosomal RNA (rRNA) are involved as accessory components in the translation process. Most of the DNA in a genome does not code for genes and is aptly referred to as intergenic or non-coding DNA. Recently, another sub-class of RNA called microRNA (miRNA) has been identified as being transcribed from this non-coding region. MicroRNAs are short sequences of approximately 20 nucleotides in length that have been shown to be correlated with human cancers. In healthy cells, there is a balance between genes that promote cell proliferation and tumourigenesis and those that suppress such processes. MicroRNAs have been shown to regulate a number of genes that have been implicated in playing a role in the development of human cancers. The staggering number of human patients afflicted with cancer and the convenient average sequence length of these miRNA has provided a significant impetus of utilizing microRNA probes in biosensor development 21, 22.

Another permutation in biosensor design has been to immobilize peptide nucleic acids (PNA) and search for DNA or mRNA targets. PNA is a DNA analog where the phosphate-deoxyribose backbone is replaced by amino acid residues 23. It has been shown that single stranded PNA exhibits an increased affinity for DNA strands in comparison to DNA itself 24. In DNA-DNA interactions the dianionic phosphates on each strand repel each other. This demonstrates why mismatched strands with decreased hydrogen bonding are energetically unstable and cannot maintain the duplex configuration. In PNA binding, this effect is absent and a more stable duplex is produced enabling improved discrimination between SNPs 24.
Solid-Phase Oligonucleotide Immobilization and Hybridization

Immobilization of oligonucleotide probes has been accomplished in a number of ways to fabricate a reusable biosensing surface. Typically, optical transduction methods utilize fused silica and its derivatives as a substrate and covalently immobilize nucleic acid material directly. This is classically done by using the epoxide terminated silane, 3-glycidoxypropylsilane (GOPS), or the amine terminated silane, 3-aminopropyltrimethoxysilane (APTMS)\(^{25}\). Both these compounds form siloxane bonds with the oxidized surface of the silicon material and adjacent APTMS or GOPS molecules. Usually coating the surface with these materials results in multilayer formation as the silane molecules polymerize together. However, monolayers have been achieved by careful control of reaction conditions\(^{25}\). Terminal amines act as nucleophiles to form covalent attachment to oligonucleotides with a free electrophile – typically a carboxylic acid. Alternatively, epoxide residues have served as electrophiles for probe nucleic acid modified with nucleophiles such as amines.

Proteins such as avidin, streptavidin and neutravidin have also been immobilized on predominantly silicon-type surfaces\(^{6,\,26,\,27}\). This is typically done by first functionalizing the surface with a terminal amine or carboxylic acid. Amino acid side chains of these proteins can be reacted with these functional groups to form stable amide bonds\(^{28}\). Immobilized avidin and derivatives have been shown to form an extremely stable non-covalent attraction with the small molecule biotin such that it is essentially irreversible\(^{6}\). Very stable immobilization chemistry has been achieved by labeling probe nucleic acid material with biotin and linking to an avidin coated surface.
Another approach to immobilization utilizes the natural affinity of thiol compounds for gold and other metallic surfaces. Oligonucleotides that are end labeled with a thiol moiety will self assemble onto gold surfaces forming a very stable metal-thiol linkage. This approach is much more common in piezoelectric, electrochemical or SPR transduction methods because the methods do not require the immobilization surface to be transparent to light.

Regardless of the method of immobilization, the local environment experienced during hybridization between immobilized probe and target is strongly affected by the density of probe sites on a surface and by the surface itself. Target that approaches an immobilized probe strand is electrostatically repelled due to the negative charge on the phosphodiester backbone of nucleic acids, which hampers duplex formation at the surface. Furthermore, as hybridization of target and probe material accumulates at the surface, the surface acquires a higher cation concentration compared to bulk solution. Finally, adjacent probe DNA can sterically hinder approaching target if the probe density is high enough. High density is considered as a 50 Å centre-to-centre strand separations for oligonucleotide probes. Higher immobilization probe densities have been found to improve the selectivity between fully complementary targets and SNPs at the expense of hybridization efficiency. This behaviour has been thought to occur because a mismatch creates a bulge in the duplex that cannot be accommodated by the density of adjacent neighbours. Therefore, gains in selectivity can be achieved by utilizing solid-phase in comparison to solution-solution phase systems. In a solid-phase system, it is also facile to establish an on-board control; the signal generated from non-selective adsorption of materials can be subtracted by implementation of reference sensors. Moreover, utilizing immobilized probes on a surface enables restoration of the sensing surface after each
hybridization event compared to solution-phase systems that have to be discarded after each use.

**Amplification**

For the majority of nucleic acid detection techniques, low target concentrations require amplification to produce a signal. Polymerase chain reaction (PCR) has revolutionized molecular biology by *in vitro* replication of potentially millions of copies of template DNA and has been fundamental to most nucleic acid detection technologies. Essentially the technique begins with heating a short region of double stranded DNA desired for amplification to the point of dissociation. The system is then cooled and two different single stranded RNA primers (~10 – 20 base pairs) are then added to hybridize to complementary regions on the 3’ ends of each single strand. Addition of the *Taq* DNA polymerase enzyme which is stable to thermal conditions facilitates replication (5’ – 3’ direction) of each single strand in the presence of the 4 nucleotide triphosphate monomers at 72 °C. At the end of one cycle of replication two new strands are created and a new cycle can be repeated after addition of more primer and appropriate thermal adjustment. Amplification occurs exponentially over periods up to several hours as 2 copies are yielded per template strand. Unfortunately, PCR has a well-earned fastidious reputation because everything has to be precisely controlled for successful amplification. For example, low dissociation temperatures result in lower copy yields since primers are blocked from binding and prolonged higher temperatures result in compromising the enzyme fidelity. Annealing temperatures that are too low allow non-specific amplifications while higher temperatures limit product yields. Finally, poorly synthesized
primers or improperly selected primer sequences may amplify any contaminating sequences present restricting confidence that presence of a certain target is genuine. These are a few examples of the more significant errors accompanying PCR. Despite these complications, PCR is readily adaptable to microfluidic devices and has been extensively combined with nucleic acid assays. Real-time PCR for example enables amplification of target nucleic acid while simultaneously quantifying the number of copies amplified using fluorescence 33. The number of copies produced is indicative of the number of target strands present in the original sample. Typical real-time PCR experiments can perform a quantitative genetic analysis in less than 30 minutes from sample preparation to signal acquisition 33. PCR can be integrated with nucleic acid biosensor platforms that offer improvements on real-time PCR methods. Integration with microfluidics based sensors would facilitate easy dispensing of amplified target to immobilized hybridization arrays creating a rapid and reusable detection method that could offer improvements in sensitivity and selectivity over traditional real-time PCR.

Developing devices that eliminate the need for amplification improves throughput and simplifies analysis. To migrate away from this dependency on amplification, sensors will need to offer technologies with very low limits of detection. Typically this is accomplished by labeling target material so the signal can be amplified instead of the amount of target. The next section will consider a variety of approaches towards development of sensors that remove the need for amplification.

**Transducers**
Most biosensing devices share a general format where a sensitive layer that immobilizes the recognition layer is interfaced to electrochemical, piezoelectric or optical transduction elements.

Electrochemical transducers involve immobilizing single stranded probe sequences to the surface of an electrode and then measuring a change in the current, voltage or capacitance of the system once the sample has been introduced. The hybridization reaction has been observed by using labels, or by label free methods.

In methods using labels, redox-active metal complex indicators such as Ru(NH$_3$)$_6^{3+}$, Co(phen)$_3^{3+}$, or ferrocene bind to the major or minor grooves or intercalate between the bases of double stranded DNA $^{34}$. Alternatively, the incoming target DNA is labeled with organic compounds such as methylene blue or is labeled with an enzyme that can continuously convert substrate into a measurable product $^{29}$. In both cases a redox signal is generated when the compound is brought close to an electrode.

In label free methods, the signal is generated when the analyte contacts the electrode as when a guanine nucleobase is oxidized. The magnitude of current flow is then indicative of the quantity of target material. Another way is to observe the change in the capacitance/resistance at the interface as hybridization occurs. For simplicity and cost-reduction, label free methods are more favorable and offer the most attractive means of meeting commercial demands.

Included under label free methods are some applications of carbon nanotubes, which have been investigated as working electrode materials for biosensors. Carbon nanotubes are basically a sheet of sp$^2$ hybridized carbon atoms arranged in hexagon rings with the ends of the sheet folded together to form a cylinder. The diameter of the cylinders is typically several nanometers and the length of the tubes is on the order of
about one micrometer. In the case of single-walled carbon nanotubes (SWNTs), all the atoms of the nanotube are located on the surface so any small fluctuations in the charge of the environment can drastically modify the electrical properties of the nanotube making them very sensitive electrodes. In addition, the all-carbon composition of nanotubes makes them highly amenable to being readily functionalized with a biorecognition element. Alternatively, it has been demonstrated that single stranded DNA interacts non-covalently with SWNTs by wrapping around the nanotube by aromatic interactions. Complementary target can then bind to the probe DNA and promote direct electron transfer between the SWNT and the target.

A new generation of miniaturized electrochemical sensors have been recently developed which utilize field effect transistor (FET) technology. The sensor is very similar to ion selective FETs in that it monitors the charge in the analyte solution. When single stranded probes immobilized to the surface gate hybridize with target DNA, the negatively charged phosphate backbone causes a change in the potential between the gate and source which changes the conductivity of the channel of the FET. The change in the conductivity can be monitored to determine the concentration of a particular target in solution.

The electronic properties of carbon nanotubes have also been exploited in the fabrication of FET devices (NTFET) in two different designs. One method utilizes a single carbon nanotube as the conducting channel between the source and the drain electrodes. The second method uses multiple SWNTs as a network to bridge the source and the drain. Star et al. in their 2006 paper used the latter method in the investigation of a NTFET based biosensor. They demonstrated the ability to discern between the mutant form of the human HFE gene and the H63D polymorphism. HFE is associated with the iron
metabolism disease hemochromatosis. The nanotubes were functionalized with either the mutant or wild type sequence of single stranded DNA and were able to discriminate between SNPs by observing a drop in conductance between the source and drain of the NTFET upon hybridization\(^{35, 36}\). Detection was label-free and was confirmed by fluorescence labeled oligonucleotide targets. This technology offers a portable, low-cost biosensor capable of SNP discrimination with a limit of detection around 500 pg/mL.

Mass sensitive techniques like acoustic (sensitive to mass and shear plane slip) and surface plasmon resonance (SPR) sensors (sensitive to “optical mass”), are also extremely attractive as platforms for biosensor systems because they do not require the use of labeled target for signal generation. Acoustic transducers, which are also called quartz crystal microbalances (QCM), are based on the piezoelectric characteristics of quartz. When pressure is applied to quartz, an electric potential develops across its surface. Similarly, when a voltage is applied to the quartz the crystal mechanically deforms. In an AC circuit, the crystal oscillates at a frequency dependent on the material, geometry and mass of the crystal that is constant as long as those parameters remain constant\(^{37, 38}\). Coating the surface of a quartz crystal with a biorecognition layer offers a means to absorb target material, which alters the resonant frequency of the crystal. Removing the adsorbed material returns the original frequency of the crystal. In one simplification, the change in frequency can be expressed by the following equation when operating with a dried film on the resonator;

\[
\Delta f = C f^2 \Delta M / A \quad (1)
\]
Where $\Delta f$ is the change in frequency of the crystal, $\Delta M$ is the change in mass of the crystal, $A$ is the surface area of the crystal and $C$ is a proportionality constant $^{38}$. 

A recent piezoelectric biosensor developed by Yao et al. in 2008 looked at the detection of genomic DNA from the hepatitis B virus $^{23}$. PNA probe was immobilized to the gold surface of a QCM and detection was confirmed observing the frequency shift caused by increasing mass at the sensor surface. Use of PNA probes in the sensor design enabled a limit of detection of 8.6 pg/L for direct detection of unlabeled genomic hepatitis B DNA without PCR amplification. Improved sensitivity was achieved by using single stranded complementary DNA labeled with the protein RecA when compared to using unlabeled DNA target. Attaching a large protein to the target material has the effect of greatly increasing the accumulated mass at the surface of the sensor per single target molecule. Comparisons of this QCM biosensor to traditional real-time PCR (RT-PCR) techniques found a 0.9613 correlation coefficient for the detection of the hepatitis B virus $^{23}$. An analysis time from sample preparation to signal measurement was quoted at less than 50 minutes. This biosensor offers a portable, low-cost technique capable of distinguishing between SNPs without PCR amplification or direct labeling. However, other techniques including real-time PCR have been found to offer reaction times significantly faster than 50 minutes $^{6,33}$.

Surface plasmon resonance is an optical technique that can be applied to surface sensing of refractive index and thickness changes occurring at the interface between two materials of different refractive indexes. The majority of the research in this field has considered interfaces consisting of a metal layer and another dielectric layer. Typically this dielectric layer is a glass or fused silica waveguide where total internal reflection can occur above a critical angle ($\theta_c$). The critical angle depends on the refractive index of the
Evanescent waves are generated when light total internally reflects on a waveguide, which produces a component of light that propagates parallel to the boundary of the two refractive indexes \(^{39}\). The evanescent wave is the electric field component of this parallel light that actually passes through the interface into the surrounding medium. An evanescent wave is created at every location at the surface of the waveguide where light impinges on the waveguide during total internal reflection. An evanescent field in resonance with oscillating electrons in the conduction band of a metal in contact with a dielectric material can excite the electrons to form a surface plasmon (SP). Resonance is achieved if the angular frequency and momentum of the incoming evanescent wave is the same as the oscillating conductance \(^{39}\). An additional stipulation requires that the orientation of the electric field component of the incoming radiation be normal with respect to the metal surface plane – referred to as p-polarized light – to resonate with the moving electrons \(^{39}\). Furthermore, in order for the intensity of the evanescent field to be intense enough to generate a detectable absorption, the metal layer should be around 60 nm or less in thickness. To ensure the correct wavevector of light, the incident radiation must pass through a medium of sufficient refractive index (silicon derivatives are typical) at a certain angle. There are only a small number of angles that satisfy the resonance condition for a particular wavelength of light and metal/dielectric composition. The reflected intensity therefore decreases as this particular angle is approached because the

\[
\sin \theta_c = \frac{n_2}{n_1} \quad (2)
\]
wavevector of the evanescent wave approaches the resonance condition for the surface plasmon. When the resonance condition for formation of an SP is achieved, a minimum reflected intensity is observed.

Typical biosensor SPR experiments utilize the Kretschmann prism arrangement which consists of a prism base with a thin layer of noble metal adhered where the biorecognition chemistry is immobilized. Light undergoing total internal reflection passes through the prism and produces an evanescent field at the prism-metal interface. Material bound to the surface slightly increases the refractive index of the metal which changes the minimum absorption angle. If the reflected intensity is plotted as a function of the angle of incidence of the source, a characteristic curve is observed with a minimum found at a particular angle. The change in the angle of incidence represents the quantity of target material bound on the sensor surface.

Recent work in 2008 by Manera et al. developed a Kretschmann SPR biosensor specific for detection of the 35S promoter – a marker in genetically modified organisms. The platform was able to obtain images of thiolated oligonucleotide probes on a thin layer of gold substrate. Hybridization of target material at probe locations at the sensing surface resulted in a local increase of refractive index that could be measured by monitoring the surface reflectivity with a CCD camera. Experiments for non-specific binding of target DNA were also examined to ensure fully complementary sequence specificity. Pilarik et al. in 2007 also developed a DNA biosensor based on SPR imaging which utilized spatially patterned multilayers of metallic materials and polarization contrasts to help improve the sensitivity of typical SPR experiments. The limit of detection for 23 mer oligonucleotide probes was found to be around 100 pM compared to the 10 nM figure typical for other SPR approaches. In addition, the flow-through approach used to bring
the target material to the sensing surface enabled complete assay times of 15 minutes or less.

SPR technologies offer a simple and label-free method of detecting oligonucleotide target. The drawback of this approach and any other optical mass-sensitive technique is that there is no guarantee that the signal being monitored is strictly due to target binding. Fluorescence methods improve confidence that signal is generated only from fluorophore labeled targets or double stranded intercalating dyes. The next sections will consider the theory behind fluorescence techniques and the most promising approaches made towards SNP analysis.

**Fluorescence Principles**

The intensity of fluorescence emission depends first on the absorption of a photon by the fluorophore. Absorption of a photon occurs in the time frame of about $10^{-15}$ s. Because this process occurs so quickly, the transition from the ground to the electronically excited state is virtually instantaneous (Franck-Condon Principle) and is called a vertical energy transition $^{41}$. The Beer-Lambert Law describes all the factors involved in this process.

$$A = \varepsilon bc \quad (6)$$

The absorbance of the chromophore (A) is the ratio of the intensity of incident light absorbed by the medium (P) to the intensity of light absorbed by only the solvent ($P_0$) $^{38,41}$. The epsilon term ($\varepsilon$) is the molar absorptivity of the chromophore, b is the length the
incident light travels through the medium and \( c \) is the concentration of chromophore in the medium\(^{38,41}\). The fluorescence intensity (\( F \)) at concentrations of fluorophore < 0.01 M is given by the following expression:

\[
F = 2.3 \Phi \varepsilon b c P_0 \quad (7)
\]

Once absorption occurs, the fluorescence intensity depends only on the quantum yield of the fluorophore. This demonstrates one advantage of using fluorescence over absorption, where simply increasing the initial intensity of the excitation source can increase the number of fluorophores in the excited state.

The emission of light from a molecule is termed luminescence and can come in two different types depending on spin multiplicity – fluorescence and phosphorescence. In a singlet excited state, an electron has a complementary spin with that of the remaining electron in the ground state. This electron can return to the ground state by emitting a photon which is termed fluorescence. If an electron in the excited state has the same spin as the remaining electron in the ground state, than the excited state is termed a triplet state. In order for the electron to return to the ground state the spin must return to the singlet state to avoid violating the Pauli Exclusion Principle\(^{42}\). This conversion is a bidirectional, low probability event referred to as intersystem crossing (ISC). Fluorescence processes are considered quantum mechanically ‘allowed’ and occur with lifetimes on the order of 10 ns\(^{38}\). Phosphorescence processes require the triplet state electron to return to the excited singlet state before emission can occur; a process which is mechanistically hindered compared to fluorescence decay and requires milliseconds to seconds\(^{38}\).
Once in the excited state, an electron can return to the ground state without the emission of a photon. Non-radiative decay is an alternate route of energy release and competes with radiative energy release. The mechanism favored will minimize the lifetime of the excited state \(^{38,41,42}\).

In both the ground and excited states there are many vibrational levels. In solution, as the distance between atoms expand and contract during vibration there is collision with the columbiaic fields of adjacent solvent molecules \(^{41}\). Upon collision, excess vibrational energy is transferred as kinetic energy to the solvent and the electron falls to the lowest vibrational level of the electronic excited state. This always occurs in fluorescence because the lifetime of the vibrational relaxation is around \(10^{-12}\) s, while fluorescence lifetime is orders of magnitude greater \(^{41}\). Additional vibrational decay results because a fluorescing molecule can decay to any of the vibrational levels of the ground state. The molecule will then rapidly decay back to the lowest vibrational level of the ground state by vibrational relaxation. Vibrational relaxation is the origin of the Stokes shift; the shift to longer wavelengths in emission than was absorbed \(^{41}\).

Internal conversion (IC) describes when a molecule passes to a lower electronic state without the emission of a photon. This increases the vibration of the molecule causing collisions with the columbiaic fields of adjacent molecules and a transfer of kinetic energy. Non-radiative decay by this mechanism is favored when there is overlap between vibrational levels associated with the ground and excited electronic state \(^{42}\).

In addition to internal mechanisms that deactivate the excited state there can also be energy transfer from the excited state molecule to the solvent or other solutes. These mechanisms are referred to as external conversion or more generally as quenching. Two
types of quenching in particular require contact between the fluorophore and quencher \(^{38, 41, 42}\); dynamic (collisional) and static quenching (only the former will be considered here).

Collisional quenching requires the quencher molecule to diffuse into contact with a fluorophore during the lifetime of the excited state. Energy is then transferred to the quencher molecule and the fluorophore returns to the ground state without the emission of a photon. The following Stern-Volmer equation describes a fluorophore undergoing collisional quenching: where \(F_0\) and \(F\) is the fluorescence in the absence and presence of quencher respectively, \([Q]\) is the concentration of quencher and \(K_D\) is the Stern-Volmer quenching constant.

\[
\frac{F_0}{F} = 1 + K_D [Q] \quad (3)
\]

\(K_D\) can be found by finding the slope of a plot of the ratio of fluorescence against the concentration of quencher \(^{41, 42}\).

A second mechanism for non-radiative decay of the excited state is through Förster Resonance Energy Transfer (FRET), the non-radiative transfer of energy from a donor fluorophore (D) to an acceptor molecule (A) in the ground state \(^{41, 43}\). This process proceeds without the emission of a photon and results from the dipole-dipole interactions between the donor and acceptor molecules. This mechanism is modified by the spectral overlap between the emission spectrum of the donor and excitation spectrum of the acceptor, the relative orientation of the absorption transition dipoles of each molecule and on the sixth power of the distance between donor and acceptor (r). The efficiency of the FRET process (E), is governed by the expression \(^{43}\),
where $R_0$ is called the Förster distance and is defined as the distance where 50% of energy transfer of the donor is transferred to the acceptor by the FRET mechanism\textsuperscript{41}. Depending on the nature of the acceptor, the molecule can then emit a photon or undergo non-radiative decay.

To reiterate, there are two mechanisms by which a fluorophore can return to the ground-state; by non-radiative or radiative decay. To what extent one process dominates compared to the other is demonstrated by the quantum yield ($\Phi$) - the ratio of emitting molecules to the total number of excited fluorophores. The quantum yield can be written as a ratio of the fluorescence rate constant ($k_r$) to the collection of all the rate constants that contribute to deactivation of the excited state, i.e. fluorescence and all the non-radiative ($k_{nr}$) decay mechanisms mentioned previously\textsuperscript{41}.

$$\Phi = \frac{k_r}{k_r + \sum k_{nr}} \quad (5)$$

Strongly fluorescing molecules will have quantum yields approaching unity and molecules that do not fluorescence to a significant extent have values near zero\textsuperscript{38}.

Fluorescence detection platforms are a sensitive, easy to use and ubiquitous transduction method that typically relies on implementation of labeled target material. In the sensor design used in this thesis, target oligonucleotide is labeled with an organic dye that accumulates at the biorecognition layer due to hybridization of target with immobilized probe. Photoluminescence from immobilized QDs is captured through FRET
processes by the label that is associated with the hybrid. The label then fluoresces, enabling quantification of the amount of target present in the analyte.

**Photoluminescence Properties of Quantum Dots**

A new class of nanocrystals has been repeatedly utilized to alleviate the shortcomings of organic dyes. Quantum dots are a class of semiconductors that are photoluminescent because they have physical dimensions typically on the order of 10 nm or less \(^{44}\). In bulk semiconductor material the energy levels are close enough to one another that they can be considered continuous bands. There are also continuous energy levels that are considered quantum mechanically ‘forbidden’ for the electron – referred to as the band gap in semiconductors. The energy band below the band gap is called the valence band and the band above the band gap is called the conduction band. By acquiring enough energy an electron may be promoted into the conduction band where the electron is now delocalized. The promotion of an electron into the conduction band leaves behind a positive hole in the valence band. The electron and the positive-hole pair are now referred to as an exciton. After around 20 ns, the electron-hole pair recombines with the emission of a photon \(^{45}\). It has been found that the electron tends to fall from the lowest energy level of the conduction band to around the highest energy level of the valence band causing the emission wavelength to roughly equal the band gap energy \(^{45}\).

Quantum dots can be described using the same concepts as bulk semiconductors with a few interesting caveats. The average physical distance between an electron in the conduction band and a hole in the valence band is referred to as the Exciton Bohr Radius and is dependent on the composition of the semiconductor. Bulk materials will far exceed
this radius but in QDs the size of the nanocrystal semiconductor approaches this length. At this size scale the energy levels of the electrons can no longer be considered continuous. The appearance of discrete energy levels within a semiconductor is referred to as quantum confinement and defines when a semiconductor is no longer considered bulk material and is instead referred to as a quantum dot \(^{44, 45}\).

The advantage most often stated in the literature for quantum dots over conventional dyes is the ability to tune the emission wavelength. By decreasing the size of the semiconductor to reach quantum confinement there are now discrete energy levels within the nanoparticle. Removing more and more atoms from the particle decreases the number of energy states available and causes the band gap energy to increase. This has the effect of increasing the energy of emission when an electron returns to the valence band demonstrating a blue shift as the number of atoms (i.e. size) in the nanoparticle decrease. By controlling the size of the quantum dot, the wavelength of emission can be tuned \(^{44}\). Additional advantages for QDs compared to organic dyes include a broad absorption profile which allows multiple dots with varying emission profiles to be excited by a single wavelength source \(^{45}\), much greater stability against photobleaching \(^{46}\) and narrower emission bands affording greater multiplexing capability without overlap \(^{44}\).

Immobilization of QDs on a surface and functionalizing the QDs with probe DNA enables detection of dye-labeled target material through energy transfer. Currently, QDs are typically used as labels in solution-phase sensors and are discarded after a single use – a costly and environmentally detrimental approach \(^{47}\). Integration of QDs into the sensor design provides access to the advantageous photoluminescent properties of QDs and also provides a route for developing a reusable QD-FRET system that could be coupled with sample-handling devices such as microfluidics or flow cells. Utilizing immobilized QDs
would also greatly improve the lifetime of the sensor compared to immobilized organic
dyes given the inherent photostability of QDs.

**Biosensors Capable of SNP Discrimination**

Discerning between fully complementary targets and targets that differ by a single
base pair while still maintaining a high enough signal to quantify the amount of target
material in a sample represents a significant challenge. There are a number of
fluorescence sensor designs to this end. Watterson et al. in 2004 developed a fiber-optic
sensing platform capable of discriminating between 19 base pair (bp) sequences differing
by a single base by carefully controlling the temperature, while also enabling fast analysis
(< 2 min) with undiminished sensor sensitivity after 80 experiments \(^{13}\). This system offers
a fast and selective means of SNP analysis but only with sequences of ~20 nucleotides
(nt). It has been shown that increasing the length of the probe sequence decreases the
energy penalty associated with a mismatch since there are a larger number of hydrogen
bonds present to stabilize the duplex.

A recent approach to detect Salmonella SNPs utilized *in situ* PCR amplification
with electrochemical transduction \(^{48}\). The system utilized two probes; one with a biotin
label and the other labeled with an antigen that binds with the target as it is amplified
during PCR. Streptavidin coated magnetic beads were added causing the amplicon to
become anchored to the bead through the biotinylated probe. The antigen probe binds to
the other end of the amplicon where an antibody labeled with a horseradish peroxidase
enzyme converts a substrate to product providing a means for current flow. The magnetic
beads served as the electrode surface and offered a way to remove the target from the
sample matrix without losing target. This system represents a very innovative and reliable way of sample cleanup and SNP analysis but it requires PCR to develop an analytical signal. The next evolution in this system would require eliminating the need for an amplification step while still maintaining enough signal-to-noise to distinguish between SNPs and fully complementary target and to remove non-specific binding. This could be difficult without the addition of another means of stringency control such as elevated temperature to remove non-complementary material. In addition, antibodies labeled with enzymes are very costly and once they are bound to an antigen they are difficult to recover.

Table 1: Transduction mechanism, a brief description and the limit of detection for a selection of nucleic acid biosensors capable of single nucleotide polymorphism discrimination. Unless otherwise noted, the signal/noise ratio for the limit of detection is taken as 3.

<table>
<thead>
<tr>
<th>Transduction Mechanism</th>
<th>Description</th>
<th>Limit of Detection with SNP discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPR imaging</td>
<td>Gold nanoparticles modified with 21mer ssDNA with SNPs and fully matched targets bound were deposited on a gold thin film-deposited glass substrate in a PDMS microchannel. SPR imaging could distinguish between fully matched and mismatched targets in fewer than 5 minutes.</td>
<td>19 fM&lt;sup&gt;49&lt;/sup&gt;</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>A polyaniline nanotube array (PANI) was deposited on a nanoscale graphite electrode. The array was then functionalized with 21mer oligonucleotide probes.</td>
<td>38 fM&lt;sup&gt;50&lt;/sup&gt;</td>
</tr>
<tr>
<td>FET</td>
<td>A label-free carbon nanotube network field effect transistor (NTNFET) device was functionalized with 12mer oligonucleotide probes to discriminate between SNPs and fully complementary targets.</td>
<td>12 pM (S/N ratio = 2)&lt;sup&gt;36&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>A microfluidics based sensor with immobilized 19mer oligonucleotide probes in a glass/PDMS microchannel was capable of discerning between</td>
<td>200 nM&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
SNPs of 202nt PCR amplicons.

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz Crystal Microbalance</td>
<td>PNA probes immobilized to a gold thin film-deposited QCM were capable of distinguishing between SNPs without the use of labels and PCR amplification.</td>
<td>8.6 pg/L^25</td>
</tr>
<tr>
<td>Fluorescence - TIRF</td>
<td>19mer oligonucleotide probes were immobilized to an optical fiber that utilized evanescent wave excitation of dye labeled targets. SNPs and fully complementary targets were discriminated by careful temperature control in less than 2 minutes.</td>
<td>0.5 – 5 nM^13</td>
</tr>
<tr>
<td>Electrochemical Enzyme Amplified</td>
<td>A PCR synthetic target was hybridized to two oligonucleotide probes; one tagged with biotin and the other with an antigen. An antibody to the antigen was then coupled with horseradish peroxidase enzyme, which creates an electrochemically active product in the presence of an added substrate.</td>
<td>2.8 fmol^48</td>
</tr>
<tr>
<td>Fluorescence – FRET</td>
<td>Conjugated polymers interacted with the negative backbone of 24mer ssDNA labeled with Flourescein dye. With duplex formation, an intercalating dye fluoresced after energy transfer from the Flourescein tag. Selectivity was maintained with careful temperature control.</td>
<td>1 nM^51</td>
</tr>
</tbody>
</table>

These examples offer a snapshot of some of the more promising technologies in this area. Table 1 includes additional examples of transduction approaches. Control of selectivity while ensuring high sensitivity is the critical concern in all technologies looking at SNPs and any competitive system will need to guarantee this.

**Nanoparticle Immobilization Methods**

Several groups have looked at laying down self-assembled monolayers of nanoparticles within microchannels. Li et al.^52^ attached gold nanoparticles to the walls of a microfluidic chip and functionalized the particles with thiol-bound probe nucleic acid for the Dengue Virus. Gold nanoparticles have been shown to have significant absorbance overlap with certain organic fluorophores. The thiolated probe molecules were found to
self-assemble in a constrained manner on the surface of the gold leading to reproducible quenching of the fluorescence of the organic dye by the gold nanoparticles. Hybridization with target led to a conformational change in the position of the dye relative to the nanoparticle surface generating a fluorescence signal that is selective for the target sequence. A study published in Langmuir in 2006 attached biotinylated probe DNA onto streptavidin coated microbeads. The microbeads were then immobilized into a microfluidic channel in a close packed configuration to enhance mass transfer from solution to the surface of the beads. Utilizing microbeads instead of the channel surface increased the surface area available for hybridization and enhanced mixing of target with the probe during fluid flow. Microbeads are also easier to modify than the actual channel surface. The limit of detection found for single stranded target was $10^{-16}$ mols and the hybridization time was found to be about several minutes. Moreover, a selectivity factor of greater than $8 \times 10^3$ was found between point mutations in ~22mer targets.

By introducing beads or nanoparticles into fluidic channels a number of innovative sensor designs can be developed to use the advantages of fluidic platforms. Within the last few years several groups have begun to immobilize semiconductor nanocrystals into microfluidic channels. Schabas et al. in 2008 have fabricated microchannels to contain cadmium selenium (CdSe) quantum dots. Microfluidics provided opportunity to control CdSe QDs in colloidal structures where the dispersal of the QDs was dependent on the polystyrene-block-copolymer found on the quantum dot surface. The quantum dot immobilization method involved depositing the QDs directly in the microchannel through adsorption. In order to build a reusable sensor the QDs will require stronger binding than achieved by adsorption in the fluidic channel to prevent sensitivity decline over progressive tests.
Immobilization of quantum dots on surfaces has included several different approaches for a number of different applications. Spin coating typically involves dissolving dots into a solution of polymer and the corresponding monomer. The surface is then covered with the solution by spin-coating the substrate followed by polymerizing the resultant film through exposure to UV-light. The layer-by-layer approach fabricates a charged polymer on the surface and then immobilizes QDs with surfaces functionalized with compounds of opposite charge to the polymer. In this manner, layers can be continually built up to produce an optically transparent film of encased quantum dots. Sol-gel approaches immobilize QDs inside of a solid matrix covered in liquid. The liquid phase is dried, and a porous material constructed from insoluble polymer remains, entrapping the QDs. Hydrogels are similar to sol-gels however the hydrated insoluble matrix is not desiccated and the QDs remain dispersed in the liquid phase. Such methods of immobilization are not suitable as sensor platforms for a number of reasons. Quantum dots entrapped in sol-gel, hydrogel or polymer matrices are not fully available to bulk solution because of solubility and diffusion, limiting target material from reaching and interacting with immobilized dots. Layer-by-layer assemblies leave only the top layer accessible to target, effectively creating a monolayer assembly that is still capable of interacting with target material.

To enhance accessibility, quantum dots have been immobilized using protein tethers linked to surfaces. Antibodies have been utilized as tethers for QDs by linking the positively charged binding domain to negatively charged quantum dots and then tethering the heavy chain of the antibody to a surface. Proteins containing specific oligopeptide sequences have a strong affinity for dots with certain shell compositions. In a similar manner, oligopeptides have also been used to link to QDs. All three of these methods
are an improvement to the methods in the previous paragraph, but still suffer from limitations. Stability of the protein-QD tether is compromised since only a single linkage can be made per QD because the QDs are of the same size scale as the protein 26. Protein tethers are also subject to conformational change and bond disruption, which limits the lifetime of the sensor. In addition, the size scale also prevents close packing of QDs along the surface limiting the density that can be achieved.

Surface chemistry that allows multiple contact sites per QD that can be covalently fabricated on typical silicon derivative substrates has been reported by Algar and Krull in 2008 26. They developed a surface chemistry that is highly resistant to denaturation and allows access to target in solution. The substrate is modified first with APTMS providing a free amine residue. The amine moiety then forms an amide bond with one of the carboxylic acid residues of 1,3,5 benzenetricarboxylic acid. This is followed by esterification of the remaining two carboxylic acids with 5-(1,2-Dithiolan-3-yl)petan-1-ol (lipoic alcohol). Lipoic alcohol has two thiol residues located at one end of the molecule and is terminated with an alcohol group at the other end. This produces four thiol residues per single APTMS molecule that can each form a stable covalent linkage with a quantum dot. Forming a linkage between the QD and the surface lipoic acid residues displaces thioalkyl acids on the surface of the QD used to make the quantum dots water soluble and creates a covalent attachment through thiol-metal affinity interactions. The QD surface once immobilized on the substrate surface is still capable of forming stable interactions with oligonucleotides or proteins 26.

**Immobilization of Nucleic Acids to Quantum Dots**
There are currently multiple methods available for functionalizing quantum dots with nucleic acids. Probe sequences with terminal thiol residues have been shown to displace thioalkyl acids on the QD surface and bind by thiol-metal interactions. Alternatively, thioalkyl acids on QD surfaces have formed amide linkages to amine terminated oligonucleotide sequences and to avidin conjugate proteins. The avidin proteins can be further functionalized with biotin labeled oligonucleotides.

Single stranded oligonucleotides immobilized onto quantum dots have significant interactions with the surface of the nanoparticle in a similar manner to nucleic acid probes immobilized onto silicon derivative substrates. In particular, when the QD surface is modified with mercaptoacetic acid or other thioalkyl acids, there is significant hydrogen bonding between the oligonucleotide and the acid moiety. The effect of the hydrogen bonding causes the nucleic acid to lie across the surface of the QD. The magnitude of adsorption between the oligonucleotide and the QD surface depends on the solution pH, ionic strength, concentration of denaturants and other factors. Increasing surface adsorption enhances the kinetics of hybridization. There is a sharpening of the melt temperature transition for oligonucleotides that adsorb strongly to QDs. Modifying surface adsorption could therefore lead to enhanced SNP discrimination.

**Microfluidics**

The move from macroscale fluid flow into the microscale marks the change from turbulent flow to laminar flow. In turbulent flow, convective mixing between adjacent streams of fluid occurs, but the only mechanism for mixing in laminar flow is diffusion. This allows finer control of mixing conditions, which could be advantageous depending on
the nature of the experiment. The Reynolds number characterizes the presence of laminar flow and is defined by the following expression,

\[ R = \frac{v_s L}{\nu} \quad (8) \]

where \( v_s \) is the mean fluid velocity, \( L \) is the characteristic length and \( \nu \) is the kinetic fluid viscosity \(^{62}\). When the Reynolds number is below \( \sim 2400 \) the system is considered to be operating under laminar flow conditions \(^{62}\). Microfluidic systems encourage laminar flow by maintaining very small channel widths (\( L \) values) that range from 100 nanometers to hundreds of micrometers \(^{27}\). Under laminar flow conditions, surface tension, frictional forces between the liquid and the channel walls and viscosity are much more significant than in macroscale fluid flow \(^{27}\). Pumping fluid through the microchannel requires overcoming these forces. Pressure driven flow and electroosmotic flow (EOF) are the two mechanisms of achieving microscale fluid flow. The flow mechanism that is used creates different fluid velocity profiles that have a consequence on how solute molecules are distributed in the channel.

Pressure driven flow utilizes fluid actuators such as syringe pumps to drive the liquid through the channel by hydrostatic pressure. The fluid mechanics of pressure-driven laminar flow state that fluid flow approaches zero towards the channel wall resulting in parabolic velocity profiles. In contrast, fluid flow by application of an electric field between each end of a channel that has surface charge creates uniform velocity profiles across the width of the channel. Electrokinetic flow will be considered in greater detail because of the engineering simplicity compared to pressure driven flow and the control it offers in manipulating nucleic acids. In electrokinetic flow, small volumes of
target nucleic acid can be delivered rapidly to the immobilized probe array for hybridization with precise control. It also enables quick removal of non-specific adsorption during signal acquisition and fast removal of target material to regenerate the sensing surface for the next experiment.

Depending on the composition of microchannel, the walls can carry a surface charge depending on the pH of the solution in the channel. Glass is a common channel material and its silanol (-SiOH) residues carry a negative charge at any pH greater than three. This promotes a compact inner layer (shear plane); an excess of positive charge that adheres close to the surface of the walls where the potential decreases linearly with distance from the wall surface. Adjacent to this wall is a diffuse counter-ion layer where the potential drops off exponentially. The grouping of charge at the surface and solution boundary is referred to as the electrical double layer. When a potential is applied across a channel, the cations in the compact inner layer move towards the cathode. Since the cations are solvated and clustered at the walls of the channel they drag the rest of the solution with them during migration - a process referred to as electroosmotic flow (EOF). The zeta potential (\(\zeta\)) is a measure of the potential at the shear plane of the double layer and is a significant predictor of the electroosmotic mobility (\(\mu_0\)) which describes the magnitude of the fluid velocity due to the EOF:

\[
\mu_0 = \frac{E\zeta \varepsilon}{\eta} \tag{9}
\]

Where \(E\) is the applied potential, \(\eta\) is the viscosity of the solution and \(\varepsilon\) is the relative permittivity.
The second mechanism for ion transport in an electric field is by electrophoresis - the movement of charged particles under the influence of a uniform electric field. The velocity of migration is given by the electrophoretic mobility ($\mu_p$), which depends on the charge on the ion ($z$), the viscosity ($\eta$) of the medium and the Stokes radius ($r$) of the ion.

$$\mu_p = \frac{z}{6\pi\eta r} \quad (10)$$

The migration direction of a solute molecule is dependent on the magnitude and direction of the electrophoretic and electroosmotic mobility. Consider a single stranded DNA strand migrating in a glass microfluidics channel. If the walls are negatively charged, positive solute ions will cluster next to the walls of the channel. When a potential is applied, the electroosmotic mobility will be in the direction of the cathode. This is the opposite direction of electrophoretic mobility that is pulling the negatively charged DNA towards the anode. The overall direction will then depend on which mobility is larger. Managing these two elution mechanisms can have an impact on how amenable the microdevice is to a particular analytical purpose.

One method of controlling the direction and speed of elution has been by modifying the zeta potential of the channel wall(s), which can be achieved by covering the channel surface with a coating. Typically with silica and glass substrates, polymers such as methylcellulose, polyethylene glycols or polyvinyl alcohols are used to either passivate the surface, change the zeta potential, or induce an alternate charge to change the direction of the EOF. Alternative methods have utilized: self-assembled monolayers (SAM) to deposit hydrocarbon functional groups by forming a siloxane bond at the
surface; laser deposition; lithography; or contact printing methods, to name a few examples 65-67.

Microfluidic channels have been constructed from a large variety of materials. However, polymer substrates have become increasingly popular. Polymer substrates include plastics, fibers and elastomers that are more durable, easier/cheaper to manufacture and are more adaptable to a particular application when compared to other materials such as silicon and its derivatives 68. Elastomers such as polydimethylsiloxane (PDMS) in particular highlight these advantages and have been used extensively as microfluidic substrates 69. PDMS can be poured into a mold at room temperature and thermally annealed after the addition of a curing agent. The molded PDMS will mirror structures found on the surface of the mold and when attached to a flat substrate, can form a microfluidic channel. Typical substrates are glass, fused silica and silicon because they are widely available and easily functionalized. The latter point is significant since a large majority of current sensing technologies modify silicon materials with the biorecognition element.

**Biosensors Utilizing Microfluidic Control**

The prevalence of biosensors that utilize microfluidic platforms has increased in literature particularly in the last several years. The majority of these sensors do not exploit the selectivity enhancements granted by these systems but rather they focus on the benefits of small sample volumes and precise delivery. Goral et al.70 have utilized an interdigitated ultramicroelectrode array (IDUA) in a glass-PDMS microchannel. The IDUA consist of anodic and cathodic pads that each has multiple nanoelectrodes. Electrodes from the
cathode overlap (interdigitate) with electrodes from the anode. A species capable of undergoing reduction and oxidization cycles between each state causing a measurable flow of electrons. This electrochemical microfluidic device was used for a highly selective analysis of RNA from the Dengue virus. A single probe was attached to liposomes containing potassium ferro/ferricyanide redox active molecules and another probe molecule was attached to a superparamagnetic bead. Single stranded targets hybridized to both of these probe sequences in a sandwich format, and the magnetic bead was captured inside the microchannel. The liposomes were then ruptured using detergent and the redox markers were oxidized or reduced on the IDUA further downstream. This system offers a very competitive ~ 1 fmol LOD and a total analysis time including incubation of about 20 minutes.

More recent work by Berdat et al.\textsuperscript{71} in 2007 reported immobilizing 38mer DNA probes on to glass surfaces located between interdigitated microelectrodes in a flow cell. Target DNA that hybridized to the probe sequences was detected without labeling by measuring the change in conductance due to counterions in the DNA backbone. A LOD of 1 nM was reported and the work also demonstrated the detection of amplified DNA from \textit{Salmonella choleraesuis} cell cultures.

A mass-sensitive microsystem has also been developed around an array of twenty microcantilevers with nucleic acid probes immobilized onto each individual cantilever\textsuperscript{72}. The group utilized a polymer microfluidic platform to deliver reagents and target to the sensing surface. Hybridization of target DNA at the sensing surface results in a mass increase on the cantilever that could be observed by an optical detection system. The system has been shown to offer sub-nanometer resolution in the displacement of the cantilever arm.
In 2007, Klostranec and coworkers reported the convergence of quantum dots and microfluidics to be used in the diagnosis of HIV, Hepatitis B and Hepatitis C viruses. The authors exploited the multiplexing advantage offered by QDs by labeling individual colored QDs with a specific antigen of the three viruses listed above. The presence of the correct antibody in human serum then binds to the antigen on the dot surface. A second antibody labeled with a fluorescent dye is specific for the human antibody and binds as in analogy to a sandwich assay. Detection of fluorescence of the dye indicated the presence of the target in the human serum, and by observing the fluorescence signature of the QD the pathogen could be identified in a manner similar to use of a barcode. Barcoding enables screening of a large number of pathogens simultaneously. Utilizing an electrokinetic microfluidic platform allows the QD barcodes to be measured sequentially as they drift across a photon counting readout, comparable to a chromatographic experiment. A nucleic acid methodology would eliminate the need for costly, disposable antibodies by replacing antigens with nucleic acid probe sequences. Furthermore, it would remove the need to synthesize or harvest antigens since nucleic acid sequences are far easier to produce.

There are a number of motivations for utilizing an electrokinetic microfluidic platform for a nucleic acid biosensor. Erickson et al. were one of the progenitors that developed an electrokinetically-controlled microfluidic genosensor. They utilized a channel structure fabricated from PDMS that was adhered to a glass substrate. The glass surface in the microchannel was first functionalized with GOPS followed by modification with 20mer oligonucleotide probes. The electrokinetic control enabled the dispensing of nanoliter sample volumes allowing for analysis times of as little as 5 minutes. In addition, once hybridization occurred, the shear gradients imposed by the EOF created a very
efficient method for the removal of any non-specific adsorption. Furthermore, the authors also reported a limit of detection of 50 pM using epifluorescence microscopy, which is competitive with techniques that require longer hybridization times. This article demonstrates a novel method of selectivity control and enables rapid equilibration of hybridization.

In addition to the electrophoretic mobility and the EOF imposed by electrokinetic control, a third means of controlling the selectivity of a microfluidic sensor is also generated. The high conductivity buffers required for electrokinetic flow and for nucleic acid hybridization create a significant rise in temperature during current flow – a phenomenon referred to as Joule heating. Ions moving towards lower potential collide with other molecules or ions in the conducting medium. The ions lose a fraction of their energy to the other constituents as kinetic energy, which manifests as an increase in the temperature of the medium. This effect is strictly dependent on the type of buffer and concentration but typically the larger the voltage applied the greater the rise in temperature. Discriminating between SNPs and fully complementary hybrids requires controlling the thermal conditions at the biorecognition layer. Generally, this has been accomplished by equipping microfluidic chips with external heaters and thermal sensors. Erickson et al. in 2005 described how the Joule heating could be controlled and more easily stabilized when using polymer/glass chips without the need for external heaters. Polymers have been found to dissipate heat rather poorly but this was compensated by the superior thermal conductivity of glass which acts as a heat sink and the large relative surface area of a microfluidics channel. By controlling the applied voltage, the amount of Joule heating could be adjusted enabling close control of the temperature conditions at the sensing surface. Chip temperatures ranging from 36 – 38 °C were
reported as being optimal for SNP discrimination when using 19-mer probes immobilized in microchannels during electrokinetic flow. When compared to the same conditions but during offline flow, optimal SNP discrimination was achieved between 40 – 45 °C \(^{16}\). This demonstrates that in addition to the Joule heating effects, the electroosmotic shear effects and electrophoretic forces contribute to inducing denaturation. In addition to the gains in selectivity, electrokinetic flow methods significantly reduce the total analysis time. High voltages allow rapid delivery of target material to probe sites for fast equilibration of hybridization reactions. Once hybridization is completed the chip can be regenerated quickly by flushing with buffer and greatly increasing the voltage. This sensor design offers manipulation of multiple factors to control the selectivity of SNPs and fully complementary hybrids and offers total reaction times in as little as 5 min \(^{16}\).

**Contributions of This Thesis**

The new work herein presents an electrokinetically driven microfluidic technique for SNP discrimination. This method immobilizes QDs onto the glass wall of a PDMS/glass microfluidics channel by surface chemistry that has been previously applied to immobilize QDs to fused silica optical fibers \(^{26}\). The surface chemistry involves modifying a silicate surface with an alkyl dithiol that can form a multidentate interaction with the shell of a quantum dot. Once immobilized, the QD serves as a scaffold for immobilizing single stranded probe DNA. Target DNA that becomes hybridized to immobilized probes can be detected either by means of a fluorescent organic dye on the target strand, or by using intercalating dyes. FRET occurs between excited QDs and the localized organic dye, inducing fluorescence of the dye. The use of QDs as excitation
sources to induce FRET decreases background because the emission wavelength for FRET can be far removed from the excitation wavelength of the QD, avoiding contributions from scatter and direct excitation of the dye. Further contributing to this sensitivity enhancement are the high extinction coefficients and quantum yields available with QDs compared to organic dyes. Coupled with this sensitivity enhancement, immobilizing QDs into microfluidic channels enables access to the benefits of electrokinetic transport. This includes fast and precise delivery of target material to immobilized probe material with additional selectivity controls not available in offline sensors. Proper manipulation of buffer and voltage conditions have been shown to discriminate between SNPs and fully complementary duplexes on the basis of thermodynamic stability \cite{14,16}. This is accomplished by exploitation of electrical, shear and thermal forces produced during electrokinetic flow. Using an EOF driven microfluidic device, target oligonucleotide can be delivered quickly and precisely to the probe DNA that is attached to QDs that are immobilized on the glass portion of the glass/PDMS microchannel. The target hybridizes with the probe in the flowing solution stream, and then virtually all adsorbed material can be removed from the sensing surface by changing the voltage conditions to increase EOF. This approach to sensor design enables additional selectivity control by dynamic change of stringency induced by changing the applied voltage, and offers moderate throughput, and improved sensitivity by use of QDs as excitation sources.
Materials and Methods:

REAGENTS AND MATERIALS

3- Aminopropyltrimethoxysilane (APTMS, 97%), Avidin hen egg white, 1,3,5 – benzenetricarboxylic acid (95%), N,N’ – dicyclohexylcarbodiimide (DCC, 99%), 4 – (dimethylamino)pyridine (DMAP, 99%), lipoic acid (99%), 3-mercaptopropionic acid (MPA, 99%), anhydrous tetrahydrofuran (THF, 99.9%), anhydrous toluene (99.8%) and tris(2-carboxy-ethyl)phosphine hydrochloride (TCEP) were from Sigma-Aldrich (Oakville, ON, Canada). Anhydrous diethyl ether, chloroform, dichloromethane (DCM), hydrochloric acid and hydrogen peroxide solution (30%) were from EMD Chemicals (San Diego, CA, USA). All reagents were reagent grade and used as received without additional purification measures. EviDot core/shell CdSe/ZnS quantum dots (Peak PL: green, 526 nm; yellow 555 nm; red 616 nm) were from Evident Technologies (Troy, NY, USA). Borosilicate Glass Beads were from Sigma (Z273627, NY, USA). Water-sensitive reagents were stored in a glovebox (LabMaster 1000, MBraun, Peabody, MA, USA) operated at better than 20 ppm water. Argon gas was from BOC Gases (BOC Canada, Mississauga, Canada). Purified water was produced by means of a Milli-Q-five-stage cartridge purification system (Millipore, Mississauga, ON, Canada): conductance was 18 MX cm. Plain glass microscope slides (7.6 cm x 2.5 cm x 1 mm) were from Fisher Scientific (Pittsburgh, PA, USA).

INSTRUMENTATION
Laser scanning confocal microscope images of immobilized QDs and Cy3 on glass microscope slides were obtained using a VersArray Chipreader (Biorad, Hercules, CA, USA) equipped with 532 and 635 nm 10mW laser sources and two detection channels. Chipreader Images were processed using Image J software.

AFM images were obtained from an Agilent Series 5500 AFM instrument (Aglinet AFM, Chandler, AZ) with a triple lock-in AC mode controller, PicoView software and NP-1 Cantilever. The instrument was capable of providing XY scanning high V noise better than 0.1 nm and Z scanning high V noise better than 0.06 nm.

Quantum dot lifetime, absorbance, emission and excitation spectra were obtained using Quanta Master PTI spectrofluorimeter (PTI, London, ON) equipped with a 75 W Xenon Arc Lamp (Ushio), R928P PMT (Hamamatsu), Dye laser (GL-302, Photon Tech), Nitrogen Laser (GL-3300, PTI) and laser dye was DPS (Exciton).

SEM images of the PDMS microchannel were obtained using an ISI-SR-50 SEM (International Scientific Instruments) equipped with a tungsten filament electron gun operated at 15 kV and an Everhart-Thornley detector. Gold sputter coating was done using an ISI PS-2 coating unit and Argon plasma. Coating was performed for 1 minute at 1.2 kV and 40 mA.

NMR spectra were obtained using a Bruker 400 MHz, 2-channel spectrometer equipped with narrow-bore liquids and an automatic sample changer.

Infrared spectra were obtained using a Nicolet Avatar 360 fourier transform infrared spectrometer.

Electrokinetic flow in microfluidics experiments was achieved using a Labsmith High Voltage Sequencer power supply (model: 3000, HVS448) equipped with eight channels of high voltage power supply (+/- 3000 V).
The plasma cleaner (model: PDC-32G) was from Harrick Scientific (Ithaca, NY, USA). The ultrasonic bath (model: 1510, 40 kHz, 85 W) was from Branson Products (Danbury, CT, USA).

PROCEDURES

Chip Fabrication

The PDMS chips used in this thesis were fabricated from a positive glass microscope slide master containing the microchannel pattern. Masters were microfabricated using a soft lithography/rapid prototyping technique developed by Duffy et al. The process involved first spin coating SU-8-5 negative photoresist on a glass slide for 30 s at 500 rpm followed by 920 rpm for 60 s. This was followed by pre-exposure hardening of the photoresist by a soft bake procedure (65 °C for 3 min and then 95 °C for 5 minutes; each with a 3 min temperature ramp time). The photoresist film was then exposed to UV radiation (Leitz, 250 W, band-pass filter 235-700 nm) through a 3500 dpi transparency mask that contained the intended channel specifications designed in AutoCAD. A post exposure bake procedure (65 °C for 1 min and then 95 °C for 2 min, each with a 3 min temperature ramp time) was used to promote additional cross-linking in the UV exposed region of the film. The unexposed photoresist was then dissolved by placing the master slide in unstirred 4-hydroxy-4-methyl-2-pentanone for 2 min followed by rinsing in distilled water. This process created a positive master with a desired microchannel pattern.
A straight-line channel structure was selected as the channel pattern with a length and width of 30 mm and 120 μm. The channel height was 8 μm and a circular injection and waste port were located at the ends of channel each 2 mm in radius. Dimensions of the channel were selected from work by Erickson et al. \(^{16}\) to produce similar conditions during electrokinetic flow.

**Cleaning of Glass Microscope Slides**

Glass microscope slides were immersed in a ~ 85 °C 5:1:1 v/v solution of deionized water, 30% NH\(_4\)OH (aq) and 30 % H\(_2\)O\(_2\) (aq) respectively for 15 minutes. Slides were then thoroughly washed with deionized water. The slides were then exposed to a ~ 85 °C 5:1:1 v/v solution of deionized water, 12 M HCl (aq) and 30% H\(_2\)O\(_2\) respectively for another 15 minutes. This was followed by another wash with deionized water and a sequential wash with isopropanol, DCM and diethyl ether before storing the slides in an oven at 112 °C.

**Modification of Glass Slides with APTMS**

Cleaned and dried glass slides were immersed in 250 mL anhydrous toluene containing 12 mL of APTMS and 2.5 mL of triethylamine to control pH conditions. The reaction was allowed to reflux under argon for 8 – 12 h. The solution was then decanted and slides were washed in sequence three times each with isopropanol, DCM and diethyl ether and were stored under vacuum until used.
Modification of APTMS Coated Glass Slides with 1,3,5-benzenetricarboxylic acid

Microscope slides modified with APTMS were placed in a Teflon holder and submerged completely in THF in a beaker. 4 g of 1,3,5-benzenetricarboxylic acid were added and the beaker was shaken to promote dissolution. 22 μL of Dicyclohexylcarbodiimide (DCC) was then dissolved in ~15 mL of THF and then added drop-wise to the beaker containing the slides over a period of 15-20 min with constant agitation. The reaction proceeded under further agitation for 12 – 24 hours. After the reaction the solution was decanted and the slides were washed sequentially with THF (3x), DCM (3x) and Ether (3x) and stored under vacuum.

Synthesis of 5-(1,2-dithiolan-3-yl)pentan-1-ol (lipoic alcohol)

2 mmol of lipoic acid was dissolved in 50 mL of tetrahydrofuran (THF). 10 mmol of borane dimethyl sulfide (5 fold excess) was added in another flask to 200 mL of anhydrous THF and cooled in an ice bath. The lipoic acid solution was then added dropwise to the borane solution under constant agitation. Once completed, the reaction was placed under argon and stirred at room temperature for 1-2 hours. The reaction was then refluxed for 8 – 10 hours to ensure complete reduction and then cooled to room temperature and placed in an ice bath. The reaction product was then extracted with three 50 mL portions of DCM. The DCM extract was then placed on a rotary vaporator to reduce the volume of DCM until nearly dry (Caution: complete removal of solvent produced an insoluble lipoic alcohol product). IR νmax (neat): 3360 (OH), 2930/2850 (CH) cm⁻¹. NMR 1H (CDCl3) δ: 1.2-2.0 (m), 2.4-2.5 (m), 3.1-3.3 (m), 3.5-3.7 (m) ppm.
NMR 13C (CDCl3) δ: 25.5, 29.1, 32.5, 34.8, 38.4, 40.2, 56.6, 62.7 ppm.

Modification of 1,3,5-benzenetricarboxylic Acid Coated Glass Slides with 5-(1,2-dithiolan-3-yl)pentan-1-ol (liopoic alcohol)

Slides coated with 1,3,5-benzenetricarboxylic acid were placed in a Teflon holder and immersed completely in THF. 2 mmol of lipoic alcohol were added to the THF solution and the beaker was agitated to ensure dissolution. 44 μL of DCC was dissolved in ~15 mL of THF and this solution was again added drop-wise to the THF solution containing the immersed slides. 3-4 crystals of 4-Dimethylaminopyridine (DMAP) were added to the solution and the beaker was shaken for 12-24 hours. Following the reaction the slides were washed with THF (3x), DCM (3x) and Ether (3x).

Immobilization MPA-capped CdSe/ZnS Quantum Dots to Lipoic Alcohol Functionalized Glass

A 0.5 μM solution of MPA capped CdSe/ZnS Core/Shell quantum dots with 3 μM TCEP in 100 mM Borate buffer (pH 8.5) was prepared. 10 μL of this solution was spotted onto a glass slide that had previously been functionalized with lipoic alcohol. The solution was then allowed to stand for 3-4 hours in a humid environment. The solution was then removed by pipette and the slide was washed several times with ddH2O and then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS) to remove any non-specifically adsorbed QDs. The slide was imaged before and after sonication using confocal microscopy.
QDs were spotted (0.5 μL) onto bare glass slides, rinsed with ddH₂O (3x) and imaged by confocal microscopy to serve as a control. The same slide was then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS) and then imaged again by confocal microscopy. The same protocol was repeated for APTMS functionalized slides. The concentration of QDs and buffer solution was the same in the controls as used above.

**Modification of APTMS Coated Glass Slides with Avidin Protein**

APTMS coated glass slides were exposed to 200 μL of a 0.5 mg/mL solution of Avidin and covered with a glass coverslip for 2 hours in a humid environment. The slides were then washed with ddH₂O (3x).

**Preparation of PDMS-glass microchannel**

Liquid PDMS casts were prepared by thoroughly mixing a base and curing agent (10:1 ratio) from a Sylgard 184 elastomer kit. The liquid PDMS was then poured over the positive master, which was subsequently cured at 65 °C for 15 minutes. This process formed a negative impression of the microchannel structure in the PDMS substrate. The injection and waste ports were then cut into the PDMS slab by hand using a punch. The PDMS elastomer was then adhered directly to a glass microscope slide modified with lipoic alcohol previously. The PDMS slabs were plasma treated just prior to binding for 30 – 60 seconds (10 MHz RF frequency) to facilitate tight sealing between the PDMS and the lipoic alcohol functionalized glass slide. The fluorescence intensity of the slide was
then obtained for comparison after the completion of the electrokinetic flow experiments. Fluorescence intensity was compared using Image J software.

**Modification of Avidin Coated Glass Slides with Biotinylated Lipoic Alcohol and MPA-capped CdSe/ZnS Quantum Dots**

A plasma treated PDMS microchannel was laid across an Avidin coated glass microscope slide. 10 μL of 0.47 mM biotinylated lipoic alcohol solution was dispensed into the injection and waste ports of the ‘I’ microchannel and the slide was placed in a humid environment for 1 hour. The solution was pulled into the channel by capillary action. The channels were then rinsed thoroughly with ddH₂O. A 0.5 μM solution of MPA capped CdSe/ZnS Core/Shell quantum dots with 3 μM TCEP in 100 mM Borate buffer (pH 8.5) was prepared. 10 μL of this solution was spotted into the injection and waste ports of the microchannel. The solution was then allowed to stand for 3-4 hours in a humid environment. The solution was then removed by pipette and the PDMS was removed from the glass slide surface. The slide was then rinsed with ddH₂O (3x) and analyzed by confocal microscopy. The slide was then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS) to remove any non-specifically adsorbed QDs and then imaged again by confocal microscopy.

QDs were spotted (0.5 μL) onto bare glass slides, rinsed with ddH₂O (3x) and imaged by confocal microscopy to serve as a control. The same slide was then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS) and then imaged again by confocal microscopy. The same protocol was repeated for APTMS
Functionalized slides. The concentration of QDs and buffer solution was the same in the controls as used above.

**Modification of Avidin Coated Glass Slides with Biotinylated Cy3 labeled DNA**

Four 0.5 μL spots of biotinylated Cy3 labeled DNA 20mer (0.5 μM in 0.1 M borate buffer) were pipetted onto Avidin coated glass microscope slides about 1 cm apart. The spots were then allowed to stand for 1.5 hours in a humid environment. The slide was then rinsed with ddH₂O (3x). The slide was then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS). Confocal images were obtained before and after sonication. The sequence used was Cy3 5’ ATT TTG TCT GAA ACC CTG T 3’ biotin, a genetic marker for the disease spinal muscular atrophy (SMA). The sequence used has no specific purpose other than being representative of a typical oligonucleotide, and was convenient because it had biotinylated fluorophore.

Biotinylated Cy3 labeled DNA was spotted (0.5 μL) onto bare glass slides, rinsed with ddH₂O (3x) and imaged by confocal microscopy to serve as a control. The same slide was then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS) and then imaged again by confocal microscopy. The same protocol was repeated for APTMS functionalized slides. The concentration of biotinylated Cy3 labeled DNA and buffer solution was the same in the controls as used above.

**Fused Silica Fiber Preparation and Cleaning**
Fibers were prepared according to a previously published protocol by Algar et al. Fibers were cut to approximately 40 mm and cladding on the fibers was dissolved in piranha solution (50% v/v 18 M H₂SO₄ and 50% v/v 30% hydrogen peroxide) over 72 hours. The fibers were washed abundantly with ddH₂O and then rinsed with acetone. Fibers were then sonicated twice in CHCl₃ for 30 min. Fused silica fibers were sonicated in a solution of glass cleaner for 10 min and rinsed several times with ddH₂O. The fibers were then cleaned according to the glass microscope slide protocol provided above and were stored in an oven at 112 °C.

Modification of Fused Silica Optical Fibers with Multidentate Thiol Ligands, QDs, Avidin and Biotinylated DNA

Fused silica optical fibers were modified with lipoic alcohol according to the APTMS-BTA-Lipoic Alcohol protocol provided above and adapted from Algar et al. To reduce the lipoic alcohol, modified fibers were immersed in a 10-20 mM aqueous solution of TCEP for a minimum of 2 hours to create free thiol residues and then rinsed with borate buffer. Modified fibers were then immersed in a 1-2 μM solution of MPA capped QDs (Peak PL: 528 nm) for 4-6 hours at room temperature. Fibers were then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS) in borate buffer. Fibers were then rinsed with borate buffer and stored in borate buffer at room temperature.

Avidin-QD conjugates were made by immersing QD modified fibers in a 0.5 mg/mL solution of Avidin in PBS for 30 – 60 min. The fibers were then rinsed with borate buffer. Fibers were then immersed in 1 μM solution of biotinylated SMN1 probe
DNA (biotin 5’ – ATT TTG TCT GAA ACC CTG T – 3’) in PBS for 15 minutes. SMN1 is a genetic marker for the degenerative disease SMA. Passivation of fibers was accomplished by immersing in a 0.5 mg/mL, pH 7.4 solution of bovine serum albumin (BSA) in 100 mM TB buffer and 20 mM NaCl for 30 mins. The fibers were then rinsed with borate buffer several times and then sonicated in 0.1 % SDS in borate buffer and rinsed again.

Hybridization of Cy3 Labeled SMN1 Target to Fused Silica Fibers Modified with QDs, Avidin and Biotinylated SMN1 Probes

Fibers that were functionalized with SMN1 probes were exposed to Cy3 labeled SMN1 target (Cy3 – 5’ ACA GGG TTT CAG ACA AAA T – 3’) or 5’ Cy3 labeled A20 as a control for 1.5 hours in PBS buffer. Fibers were then washed with PBS buffer 3x and were then sonicated (40 kHz, 85 W) for 5 minutes in 0.1% sodium dodecyl sulfide (SDS) to remove any non-specifically adsorbed material. Fibers were then imaged by the dedicated fluorescence microscopy system (see Figure 12A) that was associated with the microfluidic work bench.

Wettability Measurements on Bare Glass and Functionalized Glass Microscope Slides

Wettability measurements were collected using the following instrument and procedure. A 10.00 μL droplet of ddH₂O was deposited near the edge of a cleaned glass slide and functionalized glass slides. A light microscope that was equipped with a
protractor was placed on its side and the illumination source was placed to ensure light would travel parallel to the bench top. A sample stage supported the slide and enabled the shadow of the water droplet to be observed. Measurements were made within 1 min of deposition of water droplets and repeated at 10 other spots along the edge of the slide. Wettability measurements were performed after every successive step in a glass slide immobilization protocol.

**Electrokinetic Flow and Parabolic Flow Experiments**

A plasma treated PDMS microchannel was laid across three 0.5 μL spots of CdSe/ZnS QDs immobilized to a lipoic alcohol functionalized glass microscope slide. The channel width was approximately 1/10 the width of immobilized spot. The channel was allowed to adhere firmly to the glass surface for 30 minutes. Injection and waste ports were then filled with 0.1 M Borate buffer. A platinum wire electrode located in each port was connected to a variable high-voltage power supply. Applying a voltage between the electrodes located in each port drove solution through the channel.

Two separate electrokinetic flow conditions were based on the use of a 100 V or a 500 V potential applied between ports to drive buffer through the microfluidics channel for 10 minutes. The procedure for parabolic flow experiments connected one port to a vacuum line held at approximately 1 Torr. The vacuum was applied for 10 minutes with continuous feed of fresh solution being drawn from a solution reservoir that was attached to one port. At the conclusion of both the electrokinetic and parabolic flow experiments, the PDMS channel was removed from the surface of the slide and the fluorescence
imaging along the former location of the channel was done using a laser scanning confocal microscope.

The first control involved placing PDMS channels over immobilized spots of QDs and filling with buffer by capillary action. The PDMS channels were removed and the spot was imaged by confocal microscopy. The same APTMS and bare glass QD immobilization controls that have been mentioned above were used as comparisons in this experiment.

**QD Photoluminescent Characterization Using Spectrofluorimetry**

The absorbance, maximum excitation, emission and lifetime photoluminescent properties of Green Adirondack CdSe/ZnS MPA capped QDs (Evident Technologies, Peak PL 526) were characterized by spectrofluorimetry.

A 10 μM solution of QDs in 100 mM borate buffer and 20 mM NaCl (pH 9.25) in a 150 μL cuvette (10 mm path length) was used for absorbance, excitation and emission scans. The blank solution contained the same solution concentrations without any QDs.

Excitation scans observed emission intensity at 535 nm (counts/s) while scanning between 250 and 525 nm excitation wavelengths to find the peak excitation wavelength. A 530 Long Pass filter was used in front of the photomultiplier tube to eliminate contributions from second order diffraction.

Emission scans observed fluorescence (counts/s) between wavelengths 420 and 600 nm when using a 405 nm excitation wavelength. Emission scans for QDs required an OD1 at the excitation monochromator. The blank emission scan did not have an optical density filter.
Lifetime measurements used a 70 μL cuvette (3 mm path length) and a 0.5 μM QD solution with the same buffer concentration noted above. The instrument response factor was observed and recorded to serve as a baseline measurement for the lifetime spectrum.

Assembly of Microfluidic Optical System

![Diagram of the microfluidic optical system](image)

**Figure 1:** Schematic of microfluidic optical system capable of line scans and low-resolution images.

A microfluidic optical scanner was assembled in collaboration with W. R. Algar for analysis of microchannels during electrokinetic flow experiments. The scanner is built on a Nikon Eclipse LV150/LV150A microscope (see Figure 1). Channels constructed from PDMS and glass microscope slides can be mounted on an XYZ translation stage that enables the channel to be positioned at the focus of the objective lens.
mounted above the stage. The excitation source is a 406 nm Radius-405 diode laser (Coherent Inc., 25 mW, 4.7 x 1.6 mm elliptical beam). The output intensity can be attenuated using neutral density filters that can be placed in front of the laser and has values of 16, 8 and 4. After passing through neutral density filters, the excitation light passes through a filter cube (Chroma Technologies) equipped with an excitation filter (z405/20x), dichroic mirror (z405rdc) and emission filter. Two filter cubes are mounted in the slider on the microscope; one is capable of observing QD emission and the other for looking at FRET sensitized Cy3 emission. The dichroic mirror reflects the shorter wavelength excitation light, which passes through the objective lens to be focused onto the microchannel. This microscope is equipped with two extra-long working distance air objective lenses: A Nikon Plan Fluor ELWD 40x/0.60, DIC M/N1, infinity/0-2, WD 3.7-2.7 and a Nikon Plan Fluor ELWD 60x/0.70, DIC M, infinity/0.5-1.5, WD 2.1-1.5. Fluorescence produced from laser light excitation passes up through the same objective but is transmitted instead of reflected at the dichroic mirror to reach the photomultiplier tube.

For looking at QDs, the emission filter can be a D525/20 or D520/40 (Chroma) and for Cy3, the emission filter is either a HQ570/20 or D585/40. In both setups, the narrow bandwidth filters are used almost exclusively.
Figure 2: Picture of microfluidic optical system built from a modified Nikon Eclipse LV150/LV150A microscope.

The instrument was tested using QDs immobilized to borosilicate glass beads (see Figure 3).

Figure 3: The image is 7 borosilicate glass beads (2 mm diameter) arranged on a circle of black Playdoh mounted on a glass microscope slide. The image is 2.25 by 2.20 cm (x by y). Each bead has been modified with APTMS-BTA-Lipoic Alcohol chemistry, exposed to TCEP and 528 nm emitting MPA-QDs. The image was collected using the microfluidic optical system. (Image courtesy of W.R. Algar).
Results and Discussion

Most hybridization methods that are capable of discerning between mismatched and fully complementary target material require strict temperature and washing controls to ensure adequate selectivity but can sometimes involve several hours to complete $^1, ^{13}$. An electrokinetically controlled chip introduces Joule heating, shear and electrophoretic forces, which can be used in combination to develop selectivity that can compete with offline contemporary sensors while at the same time drastically improving the analysis time $^{14, 16}$. Selectivity for oligonucleotide sensors is described as obtaining a signal unequivocally attributed to the complementary target and with desirably no contribution from contaminating mismatched sequences. Typically, higher selectivity is achieved by depressing non-specific adsorption and by separating the thermal denaturation temperatures ($T_m$) of complementary and SNP targets. Widely separated melt temperatures demonstrate a significant difference in the thermodynamic stability of duplex formation between complementary and mismatched targets $^{78}$. Thermal denaturation temperature differences for offline sensors capable of SNP discrimination are quoted usually at around 8 – 12 $^\circ$C $^1, ^{13, 34, 48}$. Work by Erickson et al. $^{16}$ have found similar melt temperatures of around 10 $^\circ$C for electrokinetically driven microfluidic sensors with total analysis times of around 5 minutes $^{14}$.

In previous work, oligonucleotide probes have been immobilized covalently onto the inside channel surface. Typically, the channels have been constructed from a liquid PDMS elastomer poured into a mold containing a positive master of the desired channel dimensions. The elastomer is then solidified by heat-treating, removed from the mold and then covered with a glass microscope slide. A corresponding negative imprint of the
channel structure remains in the PDMS slab from the positive master creating three walls of the microfluidics channel. The remaining wall is formed by the glass microscope slide when the elastomer is adhered\textsuperscript{14, 27}. Oligonucleotide probes can be functionalized to the glass slide before or after the channel is completed\textsuperscript{27, 79}. GOPS or APTMS methods are generally used to functionalize the silicate surfaces providing a terminal epoxide or amine residue, respectively. These residues then form an attachment to a linker molecule. It has been shown that a linker creates distance between the oligonucleotide probe and increases availability of the probe molecule for hybridization\textsuperscript{80}. An oligonucleotide probe is functionalized to the end of the linker and is available for hybridization to target material delivered through the fluidic channel. Immobilizing probes directly to an inside wall of a microfluidic channel confers multiple advantages to the sensor including fast delivery of target material, and the selectivity control for electrokinetic delivery. The sample handling and flow advantages provided by electrokinetic flow also include precise thermal control through online manipulation of Joule heating conditions. Control of thermal and electrical conditions surrounding the probe sites and have been shown to allow discrimination between SNPs and fully complementary targets by exploiting different duplex stability energetics\textsuperscript{14}. Fast reaction times are also achieved by using the very small sample sizes afforded by microfluidics platforms. Physical confinement minimizes the distance required for diffusion-limited species transfer and therefore decreases the overall kinetic reaction rate.

**Quantum Dot Immobilization Strategies**
Multidentate Ligand Approach

Development of technologies that functionalize single-stranded genetic material to nanoparticles that are immobilized in microfluidic channels has been an area of increasing interest in the biosensor literature \(^3, 52, 81\). The typical reasons that have been enumerated have been the analytical advantages associated with scaling down an experiment; less analyte is required, and reagent consumption and manufacturing materials are reduced. Furthermore, processing small volumes of liquid allows rapid and controlled manipulation that enables ease of automation and can vastly improve reaction kinetics compared to traditional microarray experiments \(^{16}\). Average microarray hybridization times are on the order of 1-2 hours compared to less than 10 minutes for a microfluidics based biosensor \(^{14, 16, 27}\).

Functionalizing nucleic acid probes to QDs immobilized in microfluidic channels allows access to the advantages of microfluidic control along with the reusability of a biosensor. QDs used in solution nucleic acid assays are discarded after a single use – a significant expense compared with the cost of organic dyes \(^{47}\). Furthermore, QDs offer higher sensitivity and reusability compared with fluorescent dyes because of their enhanced quantum yields and resistance to photobleaching, respectively. CdSe/ZnS QDs have been shown to have high quantum yields and the ZnS shell has been shown to improve chemical and photobleaching stability \(^{55}\). Previous sensor designs have considered FRET occurring between organic dye-labeled target materials or intercalating dyes and immobilized QDs but in an offline capacity \(^{82-84}\). The majority of the literature has looked at QDs acting as FRET donors because of the high quantum yield and broad absorption spectrum of quantum dots \(^{85}\). Utilizing FRET instead of a single dye system
has been found to decrease background fluorescence because any acceptor dye that is not within Förster distance will not undergo fluorescence \(^{85}\). In addition, it has been found that photobleaching of organic dyes is reduced when excitation is through energy transfer prolonging the life of the fluorophore \(^{47}\). The experiment is also operated in an excitation region that lies outside the absorption spectrum of the organic dye further decreasing background fluorescence. Multiplexing with multiple QD-dye pairs is also possible because of the tunable emission bands available for quantum dots.

Immobilization of QDs onto the glass wall of microfluidic channels is currently not well developed. However, in 2008 Algar and Krull demonstrated reliable immobilization chemistry of CdSe/ZnS core/shell quantum dots to fused silica optical fibers. The QD surface once immobilized on the substrate surface is still capable of forming stable interactions with oligonucleotides \(^{26}\). This protocol has been adapted for the immobilization of QDs onto glass microscope slides used in the construction of a PDMS-glass microchannel.

The surface chemistry involves first modifying a fused silica or glass substrate with APTMS to provide a free amine residue. APTMS was selected instead of 3-Aminopropyltriethoxysilane (APTES) because less cross-linking is observed in the presence of water \(^{86}\). Water forms a bridge between the silicon atoms of adjacent APTES molecules. This cross-linking leads to multilayer formation at the substrate surface preventing reproducibility of the surface \(^{86}\). The terminal amine residue of APTMS readily forms an amide bond with one of the carboxylic acid residues of 1,3,5 benzenetricarboxylic acid (BTA). The remaining two carboxylic acid residues can then form an ester linkage with 5-(1,2-Dithiolan-3-yl)pentan-1-ol (lipoic alcohol) which is terminated with a dithiol residue. Two free carboxylic acid residues enabled a higher
density of lipoic alcohol at the surface to promote a non-covalent interaction between the terminal thiol residues and the shell of the immobilized quantum dot. Lipoic alcohol was functionalized to the surface with the terminal dithiol residues oxidized in a disulfide bridge. Before the lipoic alcohol functionalized surface was exposed to QDs, the surface was treated with the reducing agent TCEP to produce dithiol residues (see Figure 4 and 5).

**Figure 4:** Synthetic strategy used to functionalize glass surfaces with multidentate ligand films for QD immobilization.
Figure 5: Schematic of the QD immobilization and detection strategy. QD-oligonucleotide conjugates hybridize with dye-labeled target that can undergo fluorescence after energy transfer with an adjacently excited QD.
Forming a linkage between the QD and the reduced surface lipoic acid residues displaces thioalkyl acids on the QD shell surface. Thioalkyl acids were used to make the QDs water-soluble and can be exchanged with the substrate bound multidentate thiol ligand forming a metal-thiol interaction. The ZnS shell of a CdSe core QD consists of positive divalent zinc ions form ionic bonds with negatively charged divalent sulfur atoms adopting a hexagonal zinc wurtzite crystal structure. These ions form a stable association in the crystal but are capable of interacting with other molecules. Thiols have a natural affinity for ZnS because the sulfur atom in the thiol can interact in a similar manner to the $S^2$ ion in the crystal. ZnS-thiol interactions can result in either a $\text{Zn}—S_{\text{thiol}}$ interaction or an $S—S_{\text{thiol}}$ interaction. The latter are analogous to disulfide bridges but are considered much less stable than the $\text{Zn}—S_{\text{thiol}}$ bonds $^{87}$. The bond energy of the $\text{Zn}—S_{\text{thiol}}$ bond from computational studies looking at the interaction of alkyl thiols and ZnS substrates is quoted as 46.5 kcal/mol compared to 25.1 kcal/mol for bonds between the sulfur atoms of ZnS and thiol residues $^{87}$. ZnS-thiol interactions are considered to be generally weak especially when compared to the much lauded gold-thiol interaction. Theoretical and experimental results for gold-thiol interactions place the bond energy at 47 kcal/mol, which is very similar to the value of the $\text{Zn}—S_{\text{thiol}}$ interaction. It has been postulated that since the $S—S_{\text{thiol}}$ interaction is relatively weak compared to the $\text{Zn}—S_{\text{thiol}}$ interaction, any disulfide interaction would reduce the average bond strength of the ZnS-thiol interaction. It is important to note that these reactions are considered non-covalent interactions and are not as energetically significant as covalent interactions.

Algar et al. estimated the surface binding site density available for QD interaction as roughly 1 per nm$^2$, significantly less area than the 45-50 nm$^2$ that has been measured for the occupation by a single QD $^{26}$. SEM and AFM images supported this analysis, and
confirmed that the density of QDs on a fused silica surface to be on the order of $2-4 \times 10^{13}$ cm$^{-2}$. This high density suggested minimum bidentate capacity since each thiol residue was only three carbons apart on a given lipoic alcohol residue. Four thiol residues per immobilized dot was the anticipated maximum binding capacity based on the analysis of the QD immobilization density.

Algar and Krull reported that the QDs remained bound to the fused silica fibers even after sonication $^{26}$. This suggested that the rigors of the electrophoretic, electroosmotic and Joule heating forces imposed by electrokinetic flow should not remove immobilized QDs.

Before immobilization to functionalized glass slides, the CdSe/ZnS QDs were characterized by spectrofluorimetry to determine absorbance, maximum excitation, emission, and lifetime spectra (see Figures 6, 7 and 8). This information was required to ensure that the confocal microscope and microfluidic optical scanner could properly excite and capture the emitted fluorescence of the QDs.
Figure 6: Absorbance spectrum of 10 μM MPA capped QDs (Peak PL: 526 nm) suspended in a 100 mM borate and 20 mM NaCl solution buffered at pH 9.25. Spectrum was acquired using a 150 μl cuvette with a 10 mm path length. Absorbance contribution from blank was subtracted.

Figure 7: Excitation and emission spectra of 10 μM MPA capped QDs (Peak PL: 526 nm) suspended in a 100 mM borate and 20 mM NaCl solution buffered at pH 9.25. 535 nm was the wavelength collected for the excitation spectrum and 405 nm was the
excitation wavelength used for the emission spectrum. Both spectra have been corrected against signal contribution of blank solution.

**Figure 8:** Lifetime spectrum of 0.5 μM MPA capped QDs (Peak PL: 526 nm) suspended in a 100 mM borate and 20 mM NaCl solution buffered at pH 9.25.

The protocol that had been used to immobilize quantum dots through non-covalent metal-thiol interactions to fused silica fibers²⁶ was applied to the preparation of the glass wall of a microfluidic channel. Functionalized glass slides with immobilized CdSe/ZnS dots, and plain glass slides that had been exposed to QDs were both sonicated (40 kHz, 85 W) and then were examined for fluorescence intensity (see Figure 9A). Confocal fluorescence scans found significantly reduced fluorescence on slides that were not functionalized compared to the slides that were coated with lipoic alcohol (see Figure 9B). Furthermore, there was significantly greater fluorescence intensity observed when QDs were immobilized to lipoic alcohol that had been treated with TCEP then when not treated with the reducing agent.
Wettability measurements were made of the glass slide surface to characterize each immobilization step. The average wettability for each step in the glass surface modification is tabulated in Table 2.

**Table 2:** Wettability measurements in degrees of glass slides after each subsequent step in surface functionalization in the APTMS-BTA-lipoic alcohol immobilization protocol (10 data points).

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<th></th>
<th>Cleaned Glass</th>
<th>APTMS 1,3,5 benzenetricarboxylic acid</th>
<th>Lipoic Alcohol</th>
<th>TCEP</th>
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<tbody>
<tr>
<td><strong>Average Wettability Angle</strong></td>
<td>23.6 +/- 0.5</td>
<td>30.3 +/- 2.1</td>
<td>25.4 +/- 1.8</td>
<td>31.4 +/- 4.6</td>
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<td></td>
<td>29.3 +/- 3.8</td>
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**Figure 9A:** Fluorescence of immobilized QDs spotted on lipoic alcohol treated glass slides before (left column) and after (right column) sonication for 5 minutes in 0.1 % SDS. The top two rows of spots immobilized QDs on lipoic alcohol exposed to TCEP. The bottom row has QDs immobilized on lipoic alcohol that was not exposed to TCEP. The color scale bar above represents the relationship between color and fluorescence intensity in the image. The highest intensity is in the red region and lowest in the violet region with a linear intensity relationship.
Figure 9B: Spots of immobilized QDs from Figure 9A have been expressed as a histogram plot of fluorescence intensity. Fluorescence intensity of QDs immobilized to both APTMS coated slides and bare glass before and after sonication has been included as controls. All slides were rinsed several times with ddH₂O before confocal scans. For clarity, each series has been expressed as a line graph instead of a set of discrete columns.

Quantum dots immobilized to multidentate ligand functionalized glass slides were stable to sonication as was found with silica fibers. The evidence for thiol-metal affinity interactions between the QD surface and the lipoic alcohol functionalized surface is indirect. In the previous work done with the lipoic alcohol functionalized fused silica fibers, SEM and AFM images were obtained to determine the nature of the QD immobilization. The SEM images taken before and after QD immobilization showed a drastically different surface morphology. The SEM image of the fiber surface after QD immobilization appeared to be covered by a dense film of QDs as suggested by the correspondence in the size of each nodule in the image with the size of the QDs. In Figure 10, new AFM images of silica fibers demonstrate a deposition of a thin film when
observing before and after QD immobilization images. Analysis of similar AFM three-dimensional topographic images by Algar et al.\textsuperscript{26} suggest dense film coverage after a decrease in surface roughness of 1.6 +/- 0.3 nm to 0.7 nm +/- 0.1 nm was observed when moving from lipoic alcohol functionalized fibers before and after QD immobilization, respectively. TOF-SIMS analysis of the fibers after each step of the synthesis confirmed the presence of sulfur containing peaks providing confidence in the chemistry located at the fused silica surface \textsuperscript{26}. Furthermore, the most compelling evidence showed a peak photoluminescence wavelength shift when comparing dots immobilized to a surface that had undergone the final reduction step to a surface that had not. There was also a roughly 1-2 nm bathochromic shift between the mercaptopropionic acid (MPA) QDs in solution and QDs that were immobilized to fibers with reduced lipoic alcohol \textsuperscript{26}. The CdSe/ZnS QDs were capped with MPA to promote solubility in aqueous media and to facilitate interaction with hydrophilic DNA \textsuperscript{88}. The wavelength data indicated that ligand exchange occurred at the surface of the fiber between the mercaptopropionic thioalkyl ligand and the thiol residue of the surface immobilized lipoic alcohol. These findings support the presence of a metal-thiol affinity interaction between the lipoic alcohol functionalized surface and the immobilized QDs.
Figure 10: AFM images of silica fibers functionalized with lipoic alcohol exposed to TCEP before (top) and after (bottom) immobilization of QDs. The estimated QD core diameter was around 2.4 nm placing the total diameter of the nanoparticle around 3 nm. The bottom images show elongation of nodules. This was due to damage of an electronic component of the instrument that controlled scanning along one axis, and is an imaging artifact.
Surface morphology and topography are expected to be different between fused silica fibers and glass slides. Previous studies that investigated organosilane films on glass and other silicates found differences in surface morphology\textsuperscript{86}. Glass surfaces were found to contain more nodules of polymerized organosilane material with greater diversity in nodule thickness compared to surfaces with higher proportions of SiO$_2$ such as fused silica\textsuperscript{25}. Immobilization studies comparing thin films of APTMS on glass and other silicates found differences in surface morphology but still found comparable affinity of the APTMS-functionalized silicates for carboxylic acid functional groups\textsuperscript{86, 89}. Based on these studies and the clear affinity of QDs for silicates demonstrated in figure 6, the affinity of glass-modified surfaces for QDs should be comparable to fused silica surfaces.

Reproducibility of the APTMS-BTA-Lipoic Alcohol approach to QD immobilization on glass microscope slides was assessed by comparing the fluorescence intensity histogram profiles of multiple spots to that of several controls (see Figure 11). 10 QD spots (Peak PL: 528 nm) were dispensed by pipette onto glass slides functionalized with reduced lipoic alcohol. The slides were washed with ddH$_2$O and then sonicated in an aqueous 0.1 % solution of SDS and finally imaged by confocal microscopy. The results show variation between the fluorescence intensity of immobilized QDs but demonstrate a typical peak on the relative fluorescence abscissa of around 90.
Figure 11: Spots of 10 immobilized QDs on reduced lipoic alcohol functionalized glass slides have been expressed as a histogram plot of fluorescence intensity. Fluorescence intensity of QDs immobilized to both APTMS coated slides and bare glass before and after sonication has been included as controls. All slides were rinsed several times with ddH₂O before confocal scans. For clarity, each series has been expressed as a line graph instead of a set of discrete columns. Only the control spots have been included in the legend since the remaining spots all have the same chemistry.

Avidin-Biotinylated Lipoic Alcohol Approach

An alternative approach to immobilizing QDs in microfluidic channels was also attempted by first exposing an APTMS coated glass slide to Avidin protein. Carboxylic acid residues present on the surface of the Avidin protein allow amide bond formation with the surface amine residues. A PDMS microchannel was then exposed to the Avidin-
coated glass surface without plasma treatment. Dimensions of the PDMS channel used in Avidin and multidentate ligand experiments were identical and were confirmed by SEM images of the PDMS slab (see Figure 12).

![SEM images of PDMS microchannel](image)

**Figure 12:** PDMS microchannel SEM images with width and height dimensions of 120 μm and 8 μm, respectively. The ‘step’ in image A represents the height of the channel. Image B is a cross-section showing the width of the channel. The scale is provided at the bottom of each image.

Adherence between PDMS and Avidin-coated glass was found to form a tight seal that did not cause any leakage. Biotin-labeled lipoic alcohol was then introduced to both the injection port and waste port of PDMS/glass microchannel causing biotin-labeled lipoic alcohol solution to be pulled into the microchannels by capillary action. The biotin-Avidin interaction anchored the lipoic alcohol molecule to the substrate and kept the terminal multidentate ligand accessible for association with QDs. TCEP was then deposited in both channel ports to reduce the lipoic alcohol to the dithiol. Wettability measurements were used to characterize the surface between immobilization steps and are
listed in Table 3. A solution of CdSe/ZnS QDs in buffer was exposed to the reduced lipoic alcohol functionalized surface after flushing the injection and waste ports with buffer.

Table 3: Wettability measurements in degrees of glass slides after each subsequent step in surface functionalization in the APTMS-Avidin-biotinylated lipoic alcohol immobilization protocol (10 data points).

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<th>Cleaned Glass</th>
<th>APTMS</th>
<th>Avidin</th>
<th>Biotinylated Lipoic Alcohol</th>
<th>TCEP</th>
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<tbody>
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<td>Average Wettability Angle</td>
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Confocal fluorescence scans of the surface after PDMS removal was compared with confocal scans of the same slide after 5 minutes of sonication (See Figure 13A). A substantial decrease in fluorescence was observed between the two images (See Figure 13B). An experiment was performed using biotinylated DNA with a Cy3 dye label spotted on a slide surface that had been functionalized by the above method with Avidin (see Figure 14A). This was to determine if the decrease in fluorescence seen in the immobilized QD experiment was because of removal of the QDs or by removal of Avidin from the surface. The Cy3 experiment also found a substantial decrease in fluorescence before and after 5 minutes of sonication suggesting that the Avidin is being removed from the surface (see Figure 14B). It is unlikely that the Biotin-Avidin interaction is being interrupted by sonication since it has been shown to be stable under these conditions in previous studies ⁹⁰. This does not clarify if the decrease in fluorescence during QD immobilization was because of dissociation of QDs or Avidin or both from the surface. However, given the apparent instability of this QD immobilization method the Avidin
approach was abandoned in favor of the relative stability of the APTMS-BCA-lipoic alcohol approach.

**Figure 13A:** Fluorescence of QDs immobilized in a microfluidics channel before (image A) and after (image B) sonication for 5 minutes in 0.1 % SDS. The QDs were immobilized on biotinylated lipoic alcohol that was functionalized to Avidin-coated glass slides. The surface was treated with TCEP prior to exposure to QDs.

**Figure 13B:** The immobilized QDs in the injection and waste ports from Figure 13A have been expressed as a histogram plot of the fluorescence intensity. Fluorescence intensity of QDs immobilized to both APTMS coated slides and bare glass before and after sonication has been included as controls. All slides were rinsed 3x with ddH$_2$O before confocal scans.
Figure 14A: Spots of immobilized biotinylated Cy3 labeled DNA on Avidin-coated glass slides before (top image) and after (bottom image) 5 minutes of sonication in 0.1 % SDS.

Figure 14B: Spots of Cy3 labeled DNA from Figure 14A have been expressed as a histogram plot of the fluorescence intensity. Fluorescence intensity of Cy3 labeled DNA immobilized on both APTMS coated slides and bare glass before and after sonication has been included as controls. All slides were rinsed 3x with ddH2O before confocal scans.
Electrokinetic and Parabolic Flow Experiments

QDs functionalized to the glass component of glass/PDMS microchannels were exposed to an operating voltage of 500 V for 10 minutes to initiate electrokinetic flow (see Figure 15A). Fluorescence intensity measurements showed nearly complete removal of QDs from the glass slide when compared with controls (see Figure 15B). 500 V was selected based on work done by Erickson et al. It was found that a voltage range of 460 V to 525 V produced optimal signal discrimination between SNPs and fully complementary sequences. The 10-minute voltage duration was selected as being the typical duration of a complete experiment in work developed by Erickson et al. In a second interaction, experiments used 100 V applied driving potential for the same length of time. Complete removal of immobilized QDs was again observed at this lower operating voltage (data not available). It was not clear whether the shear forces induced at the walls of the fluidic channels or the electrophoretic forces were responsible for dislodging the QDs from the multidentate ligand. A high zeta potential at the shear plane of the microfluidics channel favors a higher electrophoretic mobility. The CdSe/ZnS core/shell quantum dots were capped with MPA, which contributes terminal carboxylic acid residues. The zeta potential of carboxylic acid coated CdSe/ZnS QDs has been quoted at around ~30 mV at pH 7 and increases at higher pH. A glass surface laden with quantum dots likely carries a similar zeta potential. For comparison, the zeta potential for plain glass and PDMS without plasma treatment has been measured as -88 to -66 mV and -110 to -68 mV depending on the electrolyte concentration.
Figure 15A: Examples of fluorescence from immobilized QDs spotted on lipoic alcohol treated glass slides. Image B represents the fluorescence of the immobilized QDs after exposure to electrokinetic flow at 500 V (3 spots) and parabolic flow (1 middle spot and 4 spots on the right hand side of the image). Image A is before exposure. In image B, PDMS microfluidic channels were laid across spotted QDs to introduce parabolic or electrokinetic flow. Black lines visible through each of the spots in image B represent the location of the channel. The 3 EOF exposed spots of image B have one port of the channel placed on the bottom spot and the top spot in that column does not have channel passing through it. The color scale is the same as located in Figure 9.
Figure 15B: Figure 15A histogram plot of fluorescence intensity in channels exposed to a electrokinetic flow at 500 V and parabolic flow. The control histogram represents an spot of immobilized QD that was covered with a PDMS channel and exposed to buffer during off-line conditions. The APTMS control spot represents APTMS-coated glass that was exposed to QDs and then washed 3x with ddH₂O to serve as a background comparison.

The magnitude of the zeta potential of a PDMS/plain glass microchannel of dimensions comparable to those utilized in this thesis has been quoted at around -50 to -90 mV depending on the type of buffer and concentration. Borate buffers were utilized in experiments in this thesis and were considered in previous work by Li et al along with many other buffers, albeit of lower concentration. Higher concentrations of buffer have been shown to positively increase the zeta potential. The 100 mM borate buffer used in these experiments would therefore contribute to zeta potentials in the region of -50 mV depending on how much the -30 mV potential of the QD functionalized glass influenced the overall measured zeta potential of the microchannel.
Pressure driven flow experiments were done to achieve fluid transport without electroosmotic, electrophoretic or Joule-Heating effects to determine if stripping of the QDs during electrokinetic flow was due to one or a combination of these phenomena or if the QD immobilization scheme is simply vulnerable to dissociation. Parabolic flow was introduced to a PDMS/glass fluidic channel containing QDs immobilized to reduced lipoic alcohol coated glass. The PDMS slab was then removed from the glass slide and confocal scans showed a decreased fluorescence compared to channels exposed to buffer during off-line conditions but a greater fluorescence compared to EOF channels, which showed complete absence of fluorescence compared to controls (See Figure 11B). The stability of immobilized QDs during pressure-driven flow likely reflects the parabolic profile exhibited by this method of fluid transport. The dimensions of the microfluidic channels and the viscosity of the buffers running through them favor laminar flow profiles. Laminar flow is characterized by progressively diminishing fluid velocities as the walls are approached in the channel. Therefore, the immobilized QDs at the surface would be subject to a significantly reduced force than compared to the center of the channel during parabolic flow. In contrast, in electroosmotic flow the velocity only varies across the electric double layer, from zero at the interface to a maximum at the edge. The length of the double layer is on the order of 10 nm for silica surfaces and even though the surface is functionalized, wettability data are similar to those of bare glass suggesting a basis for comparison. At this length, the thickness of the immobilized layer at the surface of the glass wall of the microchannel should be roughly the same order of magnitude. This would indicate that the immobilization layer during electroosmotic flow experiences the maximum or near-maximum velocity of flow suggesting why QDs are dislodged from the interface even under an applied voltage of 100 V. It is not known whether or not the
electrophoretic force is significant enough without the presence of the EOF to remove immobilized QDs. Experiments that observe the electrophoretic mobility of CdSe/ZnS QDs in solution while not immobilized would determine the extent of this force and therefore indicate the bond-strength of immobilization required for QDs to remain anchored to the surface.

It would therefore seem logical to reduce the zeta potential of the PDMS to suppress the EOF to a magnitude more suitable to the strength of the QD-multidentate ligand interaction. Experiments to suppress the EOF by passivating the surface with neutral coatings would interfere with the lipoic alcohol functionalization if performed on the glass slide. It has been found that the magnitude of the EOF is increased when plasma treated PDMS is used as in this experiment as part of the microchannel compared to untreated PDMS. Plasma treatment oxidizes the siloxane surface of the PDMS creating silanol residues, which increase the hydrophilicity and make the surface more amenable to generating electroosmotic flow. Using PDMS that has not been plasma treated may reduce the magnitude of the EOF assuming a reasonable seal could still be attained between the elastomer and the glass slide. Alternatively, a number of coatings are available for PDMS that can alter the EOF-influencing properties of the resultant microfluidics channel. It may be possible that reduction of the EOF may prevent dissociation of the QDs from the multidentate ligand.

Gold nanoparticles have been shown to be stable during electroosmotic flow and electrophoretic flow when non-covalently functionalized to the inside of fluidic channels typically by the lauded gold-thiol interaction. The stability granted to gold nanoparticles over quantum dots is likely due to the greater Au--S\text{thiol} bond strength compared to the ZnS-thiol interaction. An alternative explanation could be considered if it
was demonstrated that gold nanoparticles carry a smaller charge than carboxylic acid capped CdSe/ZnS QDs. Reduced surface charge has been found to significantly diminish the electroosmotic force applied to the nanoparticle \(^{52}\). Measurements of the zeta potential of plain gold nanoparticles have been quoted as -38.5 mV \(^{97}\). Amine and anion coated gold nanoparticles have been quoted as having zeta potentials as roughly -25 mV and 50-60 mV respectively \(^{98}\). The 30 mV zeta potential for CdSe/ZnS QDs is similar in magnitude to plain and amine coated gold nanoparticles. However, the anion coated gold nanoparticles are still significantly below the -68 to -110 mV magnitude quoted for non-plasma treated PDMS. Comparing the zeta potentials of the carboylic acid capped CdSe/ZnS QDs and the gold nanoparticles; it is likely that such nanoparticles would be similarly influenced by EOF. It seems that the strength of the QD-thiol interaction is the predominant effect governing the stability of QD immobilization during electrokinetic and pressure-based flow.

**Immobilization of Single Stranded DNA to Quantum Dots**

Immobilizing single stranded DNA to QDs anchored to the walls of a channel can be accomplished through non-covalent metal-thiol interactions or by covalently linking the DNA to the same shell used to fix the QDs to the glass channel. The former approach should be stable against electroosmotic flow since the shear force is not as significant at several nanometers from the wall surface. However, given that the bidentate linkage between ZnS shell dots and lipoic alcohol is not stable during EOF it is uncertain whether a unidentate thiol terminated nucleic acid ligand will remain anchored to the QD surface under electroosmotic flow conditions.
Avidin and thiol approaches have been used to functionalize QDs with nucleic acids. Probe sequences with terminal thiol residues have been shown to displace thioalkyl acids on the QD surface and bind by thiol-metal interactions\textsuperscript{26,47}. The type of thiol-metal interaction is dependent on the shell composition of the QD. In addition to thiol methods, thioalkyl acids on QD surfaces have been used to form amide linkages to amine terminated oligonucleotide sequences or to avidin conjugate proteins\textsuperscript{59,60}. Capping agents present on the surface of the QD are typically terminated with carboxylic acids to favor water solubility. The carboxylic acids can react with amine residues found on Avidin protein forming an amide bond to keep the Avidin protein attached to the surface of the QD. Avidin can then be further functionalized with biotin labeled oligonucleotides\textsuperscript{26,61}.

Fused silica fibers that had been first treated with the multidentate chemistry, followed by immobilization of QDs and then functionalization with Avidin were treated with biotinylated SMN1 probe DNA – a genetic marker for spinal muscular atrophy. The intention of this experiment was to evaluate the capability of the microfluidic optical system for detecting FRET. Fused silica fibers were used instead of glass because the chemistry of QD-Avidin conjugates has been assessed previously and was found to be stable on this surface\textsuperscript{26}. The QD-Avidin modified fibers were exposed to Cy3 labeled SMN1 target DNA (Cy3 – 5′ ACA GGG TTT CAG ACA AAA T – 3′) and Cy3 labeled A20 and then rinsed and sonicated (see Figure 16A). The latter sequence was used as a control. The microfluidic optical scanner found similar fluorescence intensity in the QD channel (see Figure 16B) for all three fibers as expected given they were exposed to the same immobilization chemistry. Conversely, the Cy3 channel showed decreased fluorescence in the middle fiber compared to the top and bottom fibers (see Figure 16C) because of reduced affinity of the biotinylated probe SMN1 oligo for the Cy3 labeled A20.
target compared to the fully complementary SMN1 target. The Cy3 target was not
directly excited; the immobilized QDs were excited using a 405 nm diode laser. These
experiments establish the capability of this microscope in detecting FRET and provide a
reasonable test for the application of this instrument in further experiments looking at
FRET in microfluidic systems.

Figure 16A: Each optical fiber in both images has been exposed to the APTMS-BTA-
Lipoic Alcohol multidentate chemistry and has been exposed to QDs (Peak PL 528 nm). The top and bottom fibers in both images have been exposed Cy3 labeled SMN1 target. The middle fiber in both images has been exposed to Cy3 labeled A20. Image A represents emission from immobilized QDs and image B represents FRET emission from Cy3 after QD excitation.
**Figure 16B:** Histogram plot of fluorescence intensity from each QD modified fiber from the image A in Figure 16A. For clarity, the histogram has been expressed as a line graph.

**Figure 16C:** Histogram plot of fluorescence intensity from each QD modified fiber from the image B in Figure 16A. For clarity, the histogram has been expressed as a line graph.
Future Work and Conclusions

An immobilization protocol that involved functionalizing fused silica optical fibers with reduced lipoic alcohol to create multidentate ligands capable of immobilizing CdSe/ZnS core/shell QDs \(^{26}\) was adapted for use on glass microscope slides. This procedure was adapted with the intention of immobilizing QDs to the glass wall of a PDMS/glass microfluidics assembly. The QDs were intended for use as a scaffold for nucleic acid probe immobilization and a FRET donor in a fluorescence transduction method that detects dye-labeled target.

The QD immobilization procedure was successfully adapted to glass and was found to be stable to sonication as confirmed by confocal fluorescence images taken before and after sonication and when compared to controls. The procedure was characterized using contact angle measurements after sequential steps in the glass slide functionalization and by AFM images taken of the glass surface after QD immobilization. Immobilization was also found to be reasonably reproducible on glass as determined from comparisons of fluorescence intensity measurements of immobilized QDs after sonication.

An alternative approach to immobilization was also tested involving first functionalizing Avidin to an APTMS coated glass slide. Biotinylated lipoic alcohol was then reduced with TCEP and reacted with QDs. However, this approach was found to be unstable when exposed to the same sonication conditions as the multidentate approach when confocal images found near-absence of significant fluorescence compared to controls. A second experiment immobilized biotinylated Cy3 labeled DNA instead of QDs to determine if the QDs were being dislodged in the biotinylated lipoic alcohol experiment or if it was Avidin that was being removed from the surface. Absence of any
significant fluorescence of Cy3 after sonication confirmed that the Avidin approach is unstable and was abandoned as an approach for immobilizing QDs into microchannels. The exact causes for the absence of QD or Cy3 fluorescence after sonication when using the Avidin immobilization method were not investigated further.

Microfluidics experiments investigated the stability of immobilized QDs by the multidentate reduced lipoic alcohol method to electrokinetic flow. QDs functionalized to the glass component of glass/PDMS microchannels were exposed to an operating voltage of 500 V for 10 minutes to initiate electrokinetic flow. Fluorescence intensity measurements showed nearly complete removal of QDs from the glass slide when compared with controls. A second set of experiments used a 100 V driving potential and again complete removal of immobilized QDs was observed.

Pressure driven flow experiments were performed to achieve fluid transport without electroosmotic, electrophoretic or Joule-Heating effects to determine if stripping of the QDs during electrokinetic flow was due to one or a combination of these phenomena or if the QD immobilization scheme is simply vulnerable to dissociation. Parabolic flow was introduced to a PDMS/glass fluidic channel containing QDs immobilized to reduced lipoic alcohol coated glass. Confocal scans showed a decreased fluorescence compared to channels exposed to buffer during off-line conditions but a greater fluorescence compared to EOF channels.

It is uncertain as to whether the difference between glass and fused silica played a significant role in the stability of the immobilized QDs and the functionalized surface. Channels constructed using fused silica slides may elucidate if the immobilized QD stability is significantly influenced by the substrate composition, or is inherent in the reduced lipoic alcohol and ZnS shell interaction. A covalent approach may prove
necessary to ensure stability of CdSe/ZnS QDs under electrokinetic flow conditions. Recent publications suggest that layer-by-layer assembly of nanoparticles may produce films that are stable in electrokinetic flow conditions and may still be accessible to target genetic material 47.

Finally, fused silica fibers that had been treated with the multidentate chemistry and then QDs, were conjugated with Avidin and then treated with biotinylated SMN1 probe DNA with the intention of evaluating the capability of the microfluidic optical system for detecting FRET. The microfluidic scanner was capable of detecting fluorescence of Cy3 that was functionalized to SMN1 target DNA when the QDs were excited with a 405 nm diode laser. These experiments demonstrated the ability of the microfluidic optical system for detecting FRET.
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