Towards The Development Of A Quantum Dot Based Bioprobe For Intracellular Investigations of Nucleic Acid Hybridization Events Using Fluorescence Resonance Energy Transfer

by

Lori Lok-Yin Chong

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Chemistry
University of Toronto

© Copyright by Lori Lok-Yin Chong (2011)
Towards The Development Of A Quantum Dot Based Bioprobe For Intracellular Investigations Of Nucleic Acid Hybridization Events Using Fluorescence Resonance Energy Transfer

Lori Lok-Yin Chong
Master of Science
Graduate Department of Chemistry
University of Toronto
2011

Abstract

The unique spectroscopic properties of quantum dots (QDs) are of interest for application in intracellular studies of gene expression. QDs derivatized with single-stranded probe oligonucleotides were used to detect complementary target sequences via hybridization and fluorescence resonance energy transfer (FRET). As nucleic acid targets are not labeled within cells, a displacement assay for nucleic acid detection featuring QDs as FRET donors was developed. QDs conjugated with oligonucleotide probes and then pre-hybridized with labeled target yielded efficient FRET \textit{in vitro}. Studies \textit{in vitro} confirmed that displacement kinetics of pre-hybridized target was a function of the stability of the initial hybridized complex. Displacement was observed as reduction in FRET intensity coupled with regeneration of QD fluorescence. By engineering the sequence of the labeled target, faster displacement was possible. The QD-probe+target system was successfully delivered into cells via transfection. Although QDs with their cargo remained sequestered in endosomal vesicles, fluorescent properties were retained.
Acknowledgments

I am grateful for the guidance of my supervisor Professor Ulrich J Krull. His enthusiasm for scientific research has been infectious. I would also like to thank Professor Scott Prosser for serving as second reader on this thesis.

I would like to extend my sincerest gratitude to all members, past and present, of the Chemical Sensors Group. Regardless of the result of my experiments, they ensured that I would leave with a smile on my face. I would like to thank Anthony Tavares, Eleonora Petryayeva, Charles Vannoy, Uvaraj Uddayasankar, Omair Noor and Russ Algar who were always willing to discuss and assist. I would like to express my appreciation for Connie Le who helped drive the in vivo aspect of this project forward in addition to our many useful and humourous discussions.

I would also like to thank the members of Dr Westwood’s lab for their many useful discussions and assistance in culturing and maintenance of cells. Their assistance ensured the success of the biological aspect of the work presented in this thesis.

In addition, I am indebted to my friends and co-workers at the Recreation and Wellness Center who ensured I maintained a balance between work and play.

Finally, I would like to acknowledge the patience and support from my friends and family. In particular, Ernest for our many vital conversations which allowed me to take a step back and see the bigger picture.
# Table of Contents

Acknowledgments .......................................................................................................................... iii

List of Tables .................................................................................................................................. ix

List of Figures ................................................................................................................................. x

List of Abbreviations ...................................................................................................................... xiii

Chapter 1 ......................................................................................................................................... 1

1 Introduction ..................................................................................................................................... 1

1.1 Biosensors versus Bioprobes .................................................................................................. 1

1.2 Nucleic Acids .......................................................................................................................... 2

1.2.1 Central Dogma of Molecular Genetics ........................................................................... 2

1.2.2 DNA Structure .................................................................................................................. 2

1.2.3 Basics of Nucleic Acid Hybridization ........................................................................... 3

1.2.4 Nucleic Acids as Bioprobes ............................................................................................ 5

1.3 Fluorescence ............................................................................................................................ 6

1.3.1 Basics of Fluorescence .................................................................................................... 6

1.3.2 Internal Processes and Non-Radiative Relaxation Mechanisms .................................... 7

1.3.3 Lifetimes and Quantum Yield ......................................................................................... 8

1.3.4 Instrumentation for Fluorescence Measurements ......................................................... 9

1.3.5 Fluorescence Resonance Energy Transfer ...................................................................... 10
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>1.4.1</td>
<td>Physical Properties of QDs</td>
</tr>
<tr>
<td></td>
<td>1.4.2</td>
<td>Achieving Biologically Compatible QDs</td>
</tr>
<tr>
<td></td>
<td>1.4.3</td>
<td>Bioconjugation with QDs</td>
</tr>
<tr>
<td></td>
<td>1.4.4</td>
<td>QDs as FRET Donors</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5.1</td>
<td>Mechanisms For Delivery</td>
</tr>
<tr>
<td></td>
<td>1.5.2</td>
<td>Endosomal Escape</td>
</tr>
<tr>
<td>1.6</td>
<td>1.6.1</td>
<td>Drosophila as Model Organisms</td>
</tr>
<tr>
<td></td>
<td>1.6.2</td>
<td>Drosophila Cell Lines as Models</td>
</tr>
<tr>
<td>1.7</td>
<td>1.7.1</td>
<td>Role of Heat Shock Proteins</td>
</tr>
<tr>
<td></td>
<td>1.7.2</td>
<td>Heat Shock Factor</td>
</tr>
<tr>
<td></td>
<td>1.7.3</td>
<td>Small Heat Shock Proteins</td>
</tr>
<tr>
<td>1.8</td>
<td></td>
<td>Summary and Contributions</td>
</tr>
</tbody>
</table>

Chapter 2  Signal Transduction of Nucleic Acid Hybridization using Quantum Dots as Fluorescence Resonance Energy Transfer Donors

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>2.2</td>
<td>Reagents and Instruments</td>
</tr>
<tr>
<td>2.3</td>
<td>Experimental</td>
</tr>
</tbody>
</table>
3.3 Experimental ................................................................................................................. 70

3.3.1 QD-DNA Probe Bioconjugates ............................................................................. 70

3.3.2 Hybridization Assays ............................................................................................ 70

3.3.3 Competitive Displacement Assays ........................................................................ 71

3.3.4 Effects of Denaturants ........................................................................................... 73

3.3.5 Measuring Lifetimes, Spectral Overlap and Förster Distances ......................... 73

3.4 Results and Discussion .............................................................................................. 74

3.4.1 Choice of Target Strands and Förster Distances ................................................... 74

3.4.2 Hybridization Assays ............................................................................................ 75

3.4.3 Displacement Assays ............................................................................................. 79

3.4.4 Effects of Formamide ............................................................................................ 86

3.4.5 Effects of Heating .................................................................................................. 89

3.5 Conclusion ..................................................................................................................... 91

Chapter 4  In vivo Analysis of the QD:probe+target Bioprobe ......................................... 94

4.1 Introduction ................................................................................................................ 94

4.2 Experimental Reagents and Instrumentation ............................................................ 98

4.3 Procedures .................................................................................................................. 100

4.3.1 Cell Culture ......................................................................................................... 100

4.3.2 Preparation of QD-DNA+target Complex .......................................................... 101

4.3.3 Cell Preparation for Transfection ........................................................................ 101
# List of Tables

Chapter 2

2.1 Oligonucleotide sequences for preliminary design of QD:probe

2.2 Kinetic parameter values for varying ratios of QD:oligonucleotide probe

2.3 Quantum yield and lifetime values for MPA ligand exchanged QDs and QD-DNA probe

Chapter 3

3.1 Oligonucleotide sequences for optimization of the QD:probe+target system

3.2 Calculated spectral overlap, Förster distance and melting temperatures for QD-DNA bioconjugates

3.3 Lifetimes and calculated FRET efficiencies for QD:probe bioconjugates hybridized to different Cy3 labeled targets

Chapter 4

4.1 Oligonucleotide sequences for QD:probe+target systems used in *in vivo* analysis

4.2 Imaging specifications
List of Figures

Chapter 1

Fig. 1.1  Nucleobase pairing
Fig. 1.2  Jablonski Diagram to illustrate absorption and fluorescence processes
Fig. 1.3  Simplified schematic of time-resolved fluorescence analysis
Fig. 1.4  Spectral Properties of QDs
Fig. 1.5  Chemical structure of mercaptopropionic acid
Fig. 1.6  Spectral Overlap
Fig. 1.7  Intracellular delivery routes

Chapter 2

Fig. 2.1  FRET response from 1 μM of Cy3 18bp-FC target sequence with varying QD to complementary probe ratios
Fig. 2.2  FRET response from 1 μM of Cy3 18bp-FC target sequence with varying QD to non complementary probe ratios
Fig. 2.3  FRET response from 1 μM of Cy3 18bp-FC target sequence with mixed probe QD: probe bioconjugates
Fig. 2.4  Dynamic range
Fig. 2.5  FRET ratios as a function of time – kinetics comparison of 1:1, 0:1 and 1:1 NC
Fig. 2.6  FRET traces for varying QD:probe ratios
Chapter 3

Fig. 3.1  **Conceptual** schematic of proposed bioprobe

Fig. 3.2  FRET response of QD:probe bioconjugate hybridized with different Cy3 labeled target sequences

Fig. 3.3  Sequence and time-dependent displacement of QD:probe+target as observed by decreasing FRET ratios

Fig. 3.4  Time based fluorescence spectra for QD:probe bioconjugates hybridized with 18bp-FC target sequence after the addition of the displacer sequence

Fig. 3.5  Time based fluorescence spectra for QD:probe bioconjugates hybridized with 18bp-3bpm target sequence after the addition of the displacer sequence

Fig. 3.6  Time based fluorescence spectra for QD:probe bioconjugates hybridized with 16bp-3bpm target sequence after the addition of the displacer sequence

Fig. 3.7  Time based fluorescence spectra for QD:probe bioconjugates hybridized with 16-3bpm target sequence after the addition of the displacer sequence in solutions of increased ionic strength

Fig. 3.8  FRET ratios of QD:probe+18bp-FC systems after the addition of the displacer sequence in the presence of varying concentrations of formamide

Fig 3.9  The effects of heating on FRET ratios for varying QD:probe bioconjugate hybridized with different target sequences

Fig 3.10  Fluorescence spectra for QD:probe bioconjugates hybridized with different target sequences after heating

Chapter 4

Fig. 4.1  Molecular structure of Hoechst 33258

Fig. 4.2  Imaging cells transfected in the presence or absence of Cellfectin® II Reagent
Fig. 4.3  Imaging cells transfected with varying concentrations of QDs

Fig. 4.4  Imaging the QD:probe+target system \textit{in vivo} and parallel controls

Fig. 4.5  Imaging of cells subjected to heat shock treatment after transfection
### List of Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A647</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CdSe/ZnS</td>
<td>Cadmium Selenide/ Zinc Sulfide core/shell</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine 3</td>
</tr>
<tr>
<td>DHLA</td>
<td>dihydrolipoic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>Hsp23</td>
<td>heat shock protein 23 (kDa = 23)</td>
</tr>
<tr>
<td>MPA</td>
<td>3-mercaptopropionic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PL</td>
<td>photoluminescence</td>
</tr>
<tr>
<td>QD</td>
<td>quantum dot</td>
</tr>
<tr>
<td>QD:probe</td>
<td>QD-DNA probe bioconjugate</td>
</tr>
<tr>
<td>QD:probe+tgt</td>
<td>QD-DNA probe bioconjugate hybridized with a labeled target strand</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>shsp</td>
<td>small heat shock protein</td>
</tr>
<tr>
<td>$T_m$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TB</td>
<td>Tris-borate</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Biosensors versus Bioprobes

Methods for biochemical analysis continue to advance in design, sensitivity, selectivity and speed. Widespread commercialization of products has transformed sensing techniques into household names; such examples include pregnancy and blood-glucose testing kits. All sensors for the analysis of biological systems feature two main components: i) a sensing component which recognizes (ideally with high sensitivity and selectivity) a particular molecule, ion or biological event, such as nucleic acid hybridization and ii) a signal transduction component which translates the function of the sensing component into a physically measurable and detectable signal. There remains a lack of clarity between the terms biosensors and bioprobes. A biosensor recognizes a specific biochemical reaction and generates a measurable analytical signal.\textsuperscript{1,2} A bioprobe is capable of performing analyses \textit{in vivo}; measuring vital functions of living organisms often in a somewhat invasive manner.\textsuperscript{1,2} It is also important to note that bioprobes are not reusable as they are not fully reversible.\textsuperscript{1} Bioprobes are not required to feature a biological sensing mechanism and could use physical transduction methods based on temperature or mass. In addition, biosensors are not confined to analytical biochemistry applications.

\textit{In vivo} analysis allows for real time examination with appropriate spatial context, and would be best achieved with bioprobes. The analysis must be sensitive and selective, considering the complex matrix. Nucleic acid detection is of particular interest as nucleic acid sequences contain a lot of genetic information. Specific sequences code for proteins that regulate and define organisms from their morphological traits to their ability to respond to different stressors.
1.2 Nucleic Acids

1.2.1 Central Dogma of Molecular Genetics

The basic paradigm of molecular genetics features deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins. DNA contains the code to all genetic information in the specific sequence of nucleotides. The basis of nucleotides are nucleobases. There are 4 different nitrogenous heterocyclic nucleobases in DNA: adenine (A), guanine (G), thymine (T) and cytosine (C). In RNA, thymine is replaced with uracil (U). There are two types of nucleobases: pyrimidines and purines. Pyrimidines are six-membered cyclic structures and include T, C and U. Purines feature a 9-membered double ring system and include A and G. A nucleobase linked with a sugar is known as a nucleoside. Through phosphoric acid ester linkages, a nucleoside is converted to a nucleotide. The primary structure of DNA is the specific sequence of the many nucleotides that are linked together. Due to the phosphate group, each nucleotide has a single negative charge. As a result, DNA as an oligonucleotide is negatively charged.

DNA resides in the nucleus of eukaryotic cells. The genetic template messages are copied into a specialized form of RNA known as messenger RNA (mRNA) in a process known as transcription. mRNA leaves the nucleus and travels through the cytoplasm to cellular structures known as ribosomes. Here through the help of other forms of specialized RNA: ribosomal RNA (rRNA) and transfer RNA (tRNA), the mRNA is translated to build a linked sequence of amino acids in a process known as translation. The final product of a protein is achieved through additional factors that help to fold and render the protein in its active conformation.

1.2.2 DNA Structure

The stability achieved from the secondary structure of two complementary strands of DNA coiled around an axis is known as a double stranded DNA (dsDNA) duplex. It is a result of base pairing based on hydrogen bonding (H-bonding) between AT and GC nucleobases. GC base pairing allows for 3 hydrogen bonds and is thus stronger than the AT interaction which features only 2. Overall, the core of the helix is hydrophobic due to the stacked nucleobases while the charged phosphate-sugar backbone ensures hydrophilicity. Although the strands are negative due to the phosphate groups, the combined strength from all the hydrogen bonds between each set of base pairs helps to overcome this electrostatic repulsion, thus stabilizing the duplex. The stability
of a DNA duplex or RNA-DNA duplex is often described by its melting temperature, \( T_m \). This is the temperature at which 50% of the strands are in the single stranded form.

DNA and RNA are both naturally synthesized nucleic acids. There also exists synthetic nucleic acids known as peptide nucleic acids (PNA) or locked nucleic acids (LNA).\(^4\,^5\) DNA and RNA utilize a 2-deoxyribose or D-ribose sugar backbone respectively while PNA features N-(2-aminoethyl)-glycine units linked by peptide bonds. PNA is of interest in the biochemical field due to its uncharged backbone.\(^5\) LNA is modified RNA where the ribose sugar is considered locked due to a methylene bridge connecting the 2’ oxygen and 4’ carbon.\(^4\) LNAs are of interest to the biochemical field as they are readily taken up by a variety of tissues and have long tissue half-lives.\(^4\)

### 1.2.3 Basics of Nucleic Acid Hybridization

The event of two complementary single strands of DNA combining to form a stable dsDNA duplex through base pairing interactions is known as hybridization. The kinetics of solution based hybridization is often described as pseudo-first ordered due to the presence of one of the strands in excess.\(^6\,^7\). Generally, one strand is referred to as the probe sequence and a corresponding complementary strand is known as the target sequence. In most cases, the target sequence is present in excess. As a result, it is capable of continually driving the reaction equilibrium towards hybridization. The event can be described using Eq. 1.1 where \( S \) represents
the fraction of unpaired oligonucleotides, \( k \) is the observed rate constant (generally in \( \text{M}^{-1} \text{sec}^{-1} \)), \( C_0 \) is the initial concentration of oligonucleotides and \( t \) is time in seconds.

\[
S = \exp(-kC_0 t)
\]  

There are several parameters which affect the rate of hybridization and thus the stability of the resulting duplex which are discussed below.

The optimum temperature for hybridization occurs at approximately 25°C below the melt temperature, \( T_m \). Salt can result in sharp changes in the melt curve (a plot of the fraction of double stranded duplexes as a function of temperature). Below 0.1 M NaCl, doubling the concentration increases the rate by a factor of 5-10. In this region, changes by a factor of 10 in NaCl concentration can result in an increase of approximately 16°C in \( T_m \). The rate continues to increase with salt concentration until it saturates at concentrations greater than 1.2 M NaCl. Increased salt concentrations result in an increased rate of hybridization for RNA-DNA duplexes but not to the same extent as for DNA-DNA duplexes. Other ions, in particular divalent cations have an even greater effect on the rate of hybridization.

The nature of the oligonucleotides is also important; the presence of mismatches can decrease the rate of hybridization by a factor of 2 for every 10% in mismatches. Mismatches disrupt the H-bonding interactions between nucleobases resulting in decreased stability of the resulting duplex. Due to the greater number of H-bonds in GC base pairing, increasing the GC content of the duplex increases in \( T_m \) as described by Eq. 1.2.

\[
T_m = 0.41(\%GC) + 69.3
\]  

The length of the strands also plays a role. When the oligonucleotides are of equal length, under standard conditions, the rate follows Eq. 1.3, indicating that the rate is proportional with length, where \( L_1 \) and \( L_2 \) refer to the average lengths of the two sequences. This is due to the presence of a large number of nucleation sites (the initiation of a base paired region).

\[
k_1(L_1) = k_2(L_2) \frac{L_1}{L_2}
\]
In cases where the length of the two sequences differs, the rate depends on the oligonucleotide in excess. When the target sequence is longer, the rate increases proportionally with the target length. However, when the probe sequence is longer there is a reduction in rate, likely due to less nucleation sites. For RNA-DNA duplexes, it is necessary to take into account the tendency of RNA to form secondary structures. As a result, when RNA is in excess, the rate observed is similar to that for DNA-DNA duplexes. In the presence of excess DNA, the observed rate for a RNA-DNA duplex is 4-5 times slower than the rate expected for a similar system of DNA-DNA duplex. The length of the resulting duplex, \( L_d \) (base pairs) also has an effect on \( T_m \). Longer duplexes are considered more stable as described in Eq. 1.4 where \( D \) is the decrease in \( T_m \) (°C).

\[
D = \frac{500}{L_d}
\]  

(1.4)

In conditions where one strand is in excess and sufficient time is given to allow complete hybridization, essentially all of the limiting strands will form duplexes. Hybridization can be measured optically by measuring the absorbance at 260 nm. Melting double stranded DNA results in increased ultraviolet (UV) absorption, known as hyperchromicity. As the duplex melts, H-bonds break and the stacking structure of the nucleobases is disrupted. This results in increased UV absorption by 20-30%. Re-annealing to form double stranded DNA produces a corresponding decrease in UV absorption known as hypochromicity. The decreased absorption is a result of the stacking of the nucleobases which leads to interactions between the π electrons of the nucleobases.

1.2.4 Nucleic Acids as Bioprobes

Nucleic acids are abundant in \textit{in vivo} environments but their abundance may also hinder analysis if detection is not selective to a certain sequence. Fortunately, it is possible to take advantage of the process of hybridization and the factors which affect \( T_m \) to achieve fine tuning of selectivity. One approach to the design of a bioprobe for nucleic acid uses the selectivity of hybridization to generate a sensitive, reliable and quantifiable signal in the event of hybridization.
1.3 Fluorescence

1.3.1 Basics of Fluorescence

Processes which involve the absorption and emission of energy in the form of light are best explained using a Jablonski diagram which illustrates the variety of energy levels and electronic states within a molecule. The singlet ground, first and second electronic states are labeled as $S_0$, $S_1$ and $S_2$ respectively and are depicted as potential energy wells. Within each electronic state exists a number vibrational energy levels labeled as $\nu_0$, $\nu_1$, $\nu_2$ etc. Within each vibrational energy level there also exist rotational energy levels (excluded from the diagram for simplicity).

![Jablonski diagram](image)

**Figure 1.2.** Jablonski diagram to illustrate the processes involved in fluorescence. (i) absorption, (ii) vibrational relaxation, (iii) internal conversion, (iv) fluorescence emission. Note that $S_1$ and $S_2$ were offset for clarity although in reality they are in the same position along the nuclear coordinate axis.

The process begins with the absorption of a photon by a molecule in its lowest energy state. Although absorption can occur with molecules at higher vibrational levels, the majority of the population at room temperature is expected to be at the lowest vibrational energy of the ground electronic state. Absorption occurs on the timescale of $10^{-15}$ sec.\textsuperscript{8,9} This is several orders of magnitude faster than nuclear motions and thus, there is no time for significant inter-nuclear
displacement. This is known as the Franck-Condon principle.\textsuperscript{8,9} As a result, absorption transitions are illustrated using straight vertical lines in a Jablonski diagram. Resonance conditions must also be satisfied for absorption to occur. The energy of the incoming photon must match the energy between $S_0$ and the excited electronic $S_n$, vibrational $\nu_n$ and rotational state that the molecule is excited to.\textsuperscript{8} Furthermore, the dipole between the incoming radiation must be aligned with the molecular transition dipole. The transition dipole moment is a vector that represents the spatial change in charge density between the ground and excited state of a molecule.\textsuperscript{9}

The emission of a photon as the molecule relaxes from $\nu_0$ of $S_1$ to $S_0$ is fluorescence. Generally, relaxation is to a higher vibrational energy level of $S_0$ followed by vibrational relaxation which occurs quickly. The energy of the photon emitted corresponds with the difference in energy between the final and initial states.

\subsection*{1.3.2 Internal Processes and Non-Radiative Relaxation Mechanisms}

After absorption, the molecule is excited to a higher energy vibrational level of an excited electronic state. Vibrational relaxation occurs quickly (on the timescale of $10^{-15}$ to $10^{-12}$ s) and energy is lost thermally resulting in the molecule existing in the lowest vibrational level of the lowest excited state.\textsuperscript{9} The localized heating is a result of collisions between the molecule and solvent molecules as kinetic energy is dissipated throughout the system. Since lifetimes are generally on the order of $10^{-8}$ seconds, fluorescence emission generally occurs from $\nu_0$ of $S_1$. This is known as Kasha’s rule.\textsuperscript{8,9}

Other non-radiative relaxation mechanisms include internal conversion and solvent effects.\textsuperscript{8,9} Internal conversion is a process where energy is conserved as the transition from $\nu_0$ of an excited electronic state $S_n$ to an isoenergetic vibrational level of a lower electronic state $S_{n-1}$. Solvent effects also play a role in the process of fluorescence. Initially, solvent molecules are arranged around the fluorophore in a manner that minimizes the potential energy of the ground state. Excitation of the fluorophore results in a new molecular dipole. Immediately after excitation (the absorption process occurs on such a fast timescale it is considered to be instantaneous) the solvent molecules will quickly rearrange themselves in order to be in a position to minimize the potential energy of the newly excited fluorophore. This occurs on a timescale of $10^{12}$ seconds.\textsuperscript{9} The difference in timescale results in a progressive decrease in energy of the excited state in a
process known as Debye relaxation. More polar solvents will have greater ability to stabilize the excited state and can lower the energy of the excited state resulting in emission at lower energies or longer wavelengths. This is known as solvatochroism. Other solvent interactions include hydrogen bonding, proton exchange and charge transfer. The combined effects of these non-radiative relaxation mechanisms contribute to the Stoke’s shift: the effect where fluorescence emission is observed at longer wavelengths and lower energies than absorption wavelengths or the energy required to excite the molecule.\textsuperscript{8,9}

1.3.3 Lifetimes and Quantum Yield

Two important characteristics of a fluorophore are its lifetime, \( \tau \), and quantum yield, \( \Phi \). The quantum yield is a measure of the efficiency of fluorescence and is measured as the ratio of number of photons absorbed to the number of photons emitted as fluorescence. This ratio encompasses the rate of fluorescence, \( k_F \) compared with the sum of all possible non-radiative decay rates, \( k_{nr} \), such as energy transfer processes, \( k_{ET} \) and quenching mechanisms, \( k_q \) as shown in Eq. 1.5.\textsuperscript{9} Intersystem crossing and \( k_{ISC} \), internal conversion \( k_{IC} \) are also non-radiative processes.

\[
\Phi = \frac{k_F}{\sum k_{nr}} = k_F \tau
\]  

(1.5)

The lifetime, \( \tau \), is a measure of the average amount of time that the excited molecule remains in the excited state and can be determined through Eq. 1.6 and 1.7. The decay in fluorescence intensity after excitation follows an exponential trend.

\[
\tau = \frac{1}{k_F + k_{nr}}
\]  

(1.6)

\[
F(t) = F_0 \exp\left(-\frac{t}{\tau}\right)
\]  

(1.7)

For a single exponential decay, 63% of the molecules have decayed from the excited state to the ground state at \( t = \tau \).\textsuperscript{8,9} For systems with multi-component lifetimes, each component is taken into consideration, where \( a_i \) is the fractional contribution of each component to the overall intensity as described in Eq. 1.8.
Most fluorophores have lifetimes on the range of 1-10 ns. It is important to note that lifetimes are independent of concentration, excitation intensity or optical pathlength unlike fluorescence intensity. However, it is very sensitive to the local environment that can affect the non-radiative relaxation pathways.

1.3.4 Instrumentation for Fluorescence Measurements

1.3.4.1 Time Domain Measurements

Time based measurements are necessary to determine $\tau$ of a particular fluorophore by plotting the logarithm of fluorescence intensity as a function of time after excitation. The sample is first excited with a pulse of light at the appropriate wavelength. The width of the pulse should be as narrow as possible ensuring that it is significantly shorter than the lifetime of the fluorophore. Time correlated photon counting (TCSPC) generates a fluorescence decay curve based on photon counts of a defined time interval after initial excitation.

The pulse source is split, part of it is sent to the sample and part of it initiates a “start” signal with the time to amplitude converter (TAC). After a preset amount of delay time, a TAC begins to charge the capacitor. When the first photon is detected, a pulse is sent to the TAC causing the capacitor to discharge. The magnitude of the discharge is proportional to the time from excitation pulse to fluorescence emission. A multi channel analyzer (MCA) can then be used to arrange the pulses received from the TAC into separate bins. The bins correspond with pre-determined brackets of time. The result is a histogram of photon counts as a function of time which illustrates the fluorescence decay pattern of the fluorophore. All molecules should have sufficient time to relax before being excited again. Generally, the delay between excitation pulses is 5-6 lifetimes. If there is insufficient time for relaxation, this can result in overlaps observed as double peaks. Pulsed excitation sources are lasers and detection is performed using a PMT.
1.3.5 Fluorescence Resonance Energy Transfer

Fluorescence or (Förster) resonance energy transfer (FRET) is a widely used application of fluorescence for diagnostics, imaging and nucleic acid based analyses. It is a distance dependent, through space Coulombic field interaction phenomenon which is fairly predictable based on the properties of the fluorophores.\(^8,9\) It is a non-radiative quenching mechanism where an excited donor fluorophore transfers its energy to an acceptor chromophore through a resonant dipolar coupling interaction. Acceptors can be quenchers that do not exhibit fluorescence or fluorophores which emit their own fluorescence upon excitation by the donor fluorophore.

Conditions for FRET require the energy difference between the ground and electronic excited states of the acceptor to be equal or less than that of the donor.\(^8,9\) Furthermore, the distance between the donor to acceptor, \(r\), taken as the magnitude of the vector separating donor and \(n\) acceptor dipoles is important. The distance at which FRET is 50% efficient is known as the Förster distance, \(R_o\). Eq. 1.9 illustrates the distance dependence of FRET where \(E\) represents the efficiency of FRET.
Spectral overlap, $J$, is a measurement of the overlap between the absorption spectra of the acceptor and emission spectra of the donor as defined by Eq. 1.10. Generally a minimum of 30% spectral integral overlap is required to observe FRET. The spectral overlap integral, $J(\lambda)$, is correlated to the number of possible resonant transitions and is a function of the fluorescence intensity of the donor, $F_D$, and the molar absorptivity of the acceptor, $\varepsilon_A$, both as functions of wavelength ($\lambda$).

$$J(\lambda) = \int \frac{F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda)d\lambda}$$

(1.10)

The Förster distance can be determined using Eq. 1.11 which describes the distance, $R_o$, as a function of several factors including the refractive index of the surrounding medium, $n$, the quantum yield of the donor, $\Phi_D$, the relative orientation between donor emission and acceptor absorption dipoles as described by the orientation factor, $\kappa^2$ and the degree of spectral resonance between the donor and acceptor as described by the spectral overlap integral $J(\lambda)$.

$$R_o^6 = \frac{9000(\ln10)\kappa^2\Phi_D J(\lambda)}{128\pi^2 N n^4}$$

$$= 8.79 \times 10^{-28} \text{ mol} \times (n^{-4}\kappa^2\Phi_D J)$$

(1.11)

The refractive index is generally assumed to be 1.4 for biomolecules in aqueous solutions. The orientation factor, $\kappa^2$, describes the relative orientation in space of the transition dipoles of the donor and acceptor. It is generally assumed to be equal to 2/3 when considering a random orientation for donors and acceptors although it can have values ranging from 0 to 4. The orientation factor can be calculated by Eq. 1.12 where $\theta_T$ is the angle between the transition emission dipole of the donor and transition absorption dipole of the acceptor. $\theta_D$ and $\theta_A$ are the angles between the dipoles and the donor-acceptor separation vector.

$$\kappa^2 = (\cos \theta_T - 3 \cos \theta_D \cos \theta_A)^2$$

(1.12)
FRET efficiency is also a function of the rate of energy transfer as shown in Eq. 1.13 where $k_{ET}$ represents the rate of energy transfer and $k_F$ is the rate of donor fluorescence.

The rate of energy transfer $k_{ET}$ is described by Eq. 1.14 where $\tau_D$ is the lifetime of the donor in the absence of any acceptors.

\[
E = \frac{k_{ET}}{k_{ET} + k_F + k^*}
\]  
(1.13)

\[
k_{ET} = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6
\]  
(1.14)

FRET efficiency, $E$, can further be defined from changes in quantum yield, $\Phi$, or lifetime, $\tau$, of the donor in the presence (subscript DA) or absence of an acceptor (subscript D) as shown in Eq. 1.15. It is important to note that the rate of energy transfer does not depend on the quantum yield or lifetime of the donor. It is sometimes calculated as a ratio of the fluorescence intensities $F_{DA}$ and $F_D$.

\[
E = 1 - \left( \frac{\tau_{DA}}{\tau_D} \right) = 1 - \left( \frac{\Phi_{DA}}{\Phi_D} \right) = 1 - \left( \frac{F_{DA}}{F_D} \right)
\]  
(1.15)

As FRET is a non-radiative decay mechanism, it competes with all other relaxation processes including fluorescence emission from the donor. As such, FRET is usually observed as a decrease in donor emission coupled with acceptor emission when the system is excited at the appropriate donor absorption wavelength.

It is important to distinguish between quenching and FRET, both of which can be visualized in part by a decrease in emission and both of which require close distance interactions. Quenching is sensitive to more physical factors such as steric shielding, contact and charge-charge interactions while FRET is a process which occurs through-space over longer distances. In quenching, the energy of the fluorophore is lost as heat while in FRET, the fluorophore transfers its energy to an acceptor. The sensitivity of FRET efficiencies to separation distance offers some practical applications to monitor distance-dependent events in biochemical analyses. For example, FRET can be used as a “spectroscopic ruler” to measure distances between sites on
proteins.\textsuperscript{9} Hybridization of two individual strands of oligonucleotides provides the proximity required for FRET.

### 1.4 Quantum Dots (QDs)

Quantum dots (QDs) are luminescent semiconductor nanocrystals with unique intrinsic photophysical properties which provide significant advantages over organic dyes for a variety of biological, medical and diagnostic applications. QDs are much more resistant to photobleaching and chemical degradation and also exhibit longer lifetimes than conventional organic fluorophores. This allows for the use of QDs to image thick cells and tissues without damaging the specimen. QDs have been observed for several hours with confocal, epifluorescent and total internal reflection microscopes.\textsuperscript{11} Previously, the task required collecting multiple optical sections, over long timescales. The longer lifetimes of QDs in comparison to molecular dyes allows for imaging capabilities using pulsed lasers and time-gated detection with significantly reduced levels of background noise.\textsuperscript{12}

There are two spectrophotometric properties of QDs that are of particular interest. One is the size-tunable symmetrical and narrow emission (full width at half maximum is generally between 25-40 nm) that varies as a function of core size and quantum confinement effects for binary combinations of semiconductors. The second is the broad excitation spectrum with increasing absorbance towards the ultraviolet (UV) range. The molar extinction coefficient of QDs is generally 10-100x greater than that of common organic fluorophores. These two properties allow for a large Stoke’s shift and multiplexing potential.

There exist shortcomings to QDs, most notably a property known as blinking where QDs randomly alternate between emitting and non-emitting states.\textsuperscript{13} This intermittent photoluminescence (PL) is observed from single QDs and can prove to be problematic for single molecule detection work. There have been reports of suppressed blinking by passivating the surface of the QDs with thiol based ligands.\textsuperscript{13} QDs will also exhibit increased fluorescence intensity after excitation, referred to as photobrightening. This property can result in difficulties for quantification studies. Both blinking and photobrightening are believed to be a result of the mobile charges on the surface of the QDs.\textsuperscript{12}
Figure 1.4. Comparison of spectral properties of (i) QDs and (ii) Cyanine 3 (Cy3), a common fluorophore. Excitation spectra are shown in blue while emission spectra are illustrated in red. Note the much larger Stoke’s shift in QDs compared to Cy3.

1.4.1 Physical Properties of QDs

QDs are generally 1-10 nm in diameter, making them much larger than most organic dye molecules. The size of QDs can cause issues in in vivo applications with concerns to clearance from the body and toxicity issues. However it is also beneficial in providing a large surface area for attachment of moieties such as proteins, peptides, oligonucleotides and other chemical molecules to a single QD. A single QD can even contain a combination of moieties to provide for multi-functionality.

QDs are generally synthesized as monodisperse semiconductor nanocrystals using CdSe, CdS or CdTe by injecting soluble precursors into an organic solvent at elevated temperatures (300°C+). Achievement of the variety of sizes and thus different PL QDs, requires adjusting the amount of precursors and/or crystal growth time. QDs synthesized in this manner have generally very low quantum yield (<10%). The addition of a shell of high band gap semiconductor material (i.e., ZnS) is epitaxially grown around the core to increase the quantum yield. This wider band gap material shell helps to passivate the core. The shell is also an important factor for biological applications as it helps to prevent leaching. A more extensive review on the physical properties photophysics of QDs can be found elsewhere.
1.4.2 Achieving Biologically Compatible QDs

As they are synthesized in organic solvents, the surface of QDs is initially decorated with hydrophobic organic ligands, most commonly trioctylphosphine (TOP) and trioctylphosphine oxide (TOPO). To render them water soluble and thus biologically relevant, there exist a wide variety of coatings including amphiphilic polymers, micellar encapsulation and surface ligands. Amphiphilic polymers coordinate with TOP/TOPO via hydrophobic interactions through long alkyl chains. The same polymer contains polar groups such as carboxylic acids to achieve hydrophilicity. Polymer coatings have been reported to offer superior brightness but also significantly increase the hydrodynamic diameter (HD) to sizes that may hinder biological applications (20-80 nm). Micellar encapsulation uses amphiphiles to achieve solubility, while stability is achieved by cross-linking the polar functionalities on the surface of the micelle. The replacement of TOP/TOPO ligands with hydrophilic ligands in a mass driven process is known as ligand exchange. The main surface chemistry used in this thesis for solubility featured the use of ligand exchange using thiol-alkyl-acids. Extensive reviews on techniques for biological compatibility can be found elsewhere.\textsuperscript{19-25}

Thiol-alkyl acid surface chemistry was chosen due to the fact that it is the most compact of surface chemistries available, is stable for extended periods of time and is easily prepared.\textsuperscript{26} The compact nature makes it ideal for FRET applications, a distance dependent phenomenon. The drawbacks of thiol-alkyl acid surface chemistry include poor stability at low pH, UV photooxidation of ligands and lower quantum yield compared to polymer coatings. The thiol-alkyl acid ligand used was mercaptopropionic acid (MPA).

![Figure 1.5. Chemical structure of MPA](image)

Ligand exchange with MPA involves the coordination of $S^-$ with Cd$^{2+}$ or Zn$^{2+}$ on the surface of the QD. The polar carboxylic acid groups are thus pointing in the direction of the solvent for solubility. Under basic conditions, the carboxylic acid groups are negatively charged. The pKa of
MPA has been reported to be 4.3\textsuperscript{27}, however when coated to nanoparticles or in thin films, the pKa of carboxylic acids can increase by several units. MPA coated QDs have been reported to have a pKa or approximately 7.8\textsuperscript{28}. The negatively charged ligands result in electrostatic repulsion minimizing the formation of aggregates and maintaining their solubility. Unfortunately, desorption of thiol-alkyl acid ligands from the surface of the QD can occur over time. This process can be slowed by storing the QDs at 4°C. Stable MPA ligand-exchanged, water soluble QDs have been achieved with a shelf life of over a year. Monothiols are believed to be more stable than bidentate ligands due to their ability to pack more densely on the surface of the QD. An additional reason for the choice of MPA was the subsequent bioconjugation of oligonucleotides to the surface of the QD featuring a competitive displacement of monothiol MPA with dithiol modified oligonucleotide probes.

1.4.3 Bioconjugation with QDs

As with the variety of ligand chemistries available, there is also a wide variety of bioconjugation techniques for the attachment of proteins, peptides, oligonucleotides and other biomolecules. Three main schemes exist. The most common method features the attachment of the biomolecule to a functional group on the modified QD via covalent modification chemistry. For example, amines modified with N-hydroxysuccinimide (NHS) esters with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) activated carboxyl groups. Electrostatic interactions can also be used. In one of the earliest examples of this method, maltose binding protein (MBP) was engineered to express a leucine domain which was positively charged. Combined with negatively charged dihydrolipoic acid (DHLA) functionalized QDs, a self-assembled complex was formed\textsuperscript{29}. The bioconjugation technique used in this thesis invokes direct interaction with the QD surface through metal-affinity interactions. Again, this strategy was chosen to minimize the distance from the surface of the QD for FRET purposes. An extensive review of various bioconjugation techniques can be found elsewhere\textsuperscript{19,22,24,25,30-35}. Surface passivation helps to reduce the leakage of excitons outside the core which in turn enhances the photochemical stability of QDs\textsuperscript{17,18,36,37}.

1.4.4 QDs as FRET Donors

The photophysical properties of QDs are conducive to development of practical FRET donors. Compared to conventional organic dyes that often exhibit significant overlap between donor and
acceptor emission, QDs have narrow, symmetrical emissions. In combination with their strong absorbance in the UV range, the possibility of directly exciting an acceptor while using QDs as donors is greatly minimized. These properties also allow for multiplexing possibilities using a single excitation source. It is also possible to increase FRET efficiencies by increasing the number of acceptors around a single QD. It has been experimentally confirmed that a value of 2/3 can be assumed for the orientation factor $\kappa^2$ in systems where both the QD and acceptor are present in random orientations. QDs have been applied as FRET donors with organic fluorophores with success in a variety of applications. The use of QDs as FRET donors with organic fluorophores in a sensing strategy was illustrated in a study for the detection of 2,4,6-trinitrotoluene (TNT) in aqueous environments. Anti-TNT specific antibody fragments were attached to QDs via metal-affinity coordination. Upon binding with dye-labeled TNT analogues, QD PL was quenched. The addition of soluble TNT was observed to displace the dye-labeled TNT analogue, eliminating FRET and a concentration-dependent recovery of QD PL. The use of QDs as FRET donors for in vivo cellular studies has also been reported in the study of transferrin-receptor (TFR) mediated endocytosis. Energy transfer was visualized by confocal microscopy between QD-Tfn donor and a fluorophore AF-Tfn acceptor conjugates during endocytic trafficking.

QDs are not effective energy acceptors when coupled with organic fluorophore donors. This is due to the direct excitation of QDs (recall their large Stoke’s shift and broad absorption spectra) as well as a large difference in excited state lifetimes between organic dyes and QDs (recall QDs have longer lifetimes). The radiative decay process of the donor outcompetes any non-radiative decay processes and no energy transfer is observed.
1.5 Intracellular Delivery

The prevalence of nanoparticles and nanotechnology has led to multiple studies towards their incorporation into cells and whole animals for tracking targets, imaging, diagnostic, therapeutic other biomedical applications.\textsuperscript{11,12,14,22,24,25,33,41,42} Cell membranes are semi-permeable structures, consisting of carbohydrates, proteins embedded in phospholipid bilayer. Cell membranes play a vital role in cell biology and are responsible of the uptake of nutrients, release of waste and communication between other cells and the environment. Small molecules such as water and oxygen are allowed to diffuse freely across the plasma membrane through the use of concentration gradients. Other ions and amino acids cross the plasma membrane through integral membrane protein pumps or ion channels. The exact method of intracellular delivery and eventual intracellular localization of nanoparticles varies depending on size, shape and surface chemistry.\textsuperscript{43} The scope of this discussion will focus on intracellular delivery as it pertains to quantum dots.

Toxicity issues are mentioned in every study but generalizations and trends are difficult to establish due to the multitude of quantum dot and bioconjugation methods utilized.\textsuperscript{12,25,41,43,44} Furthermore, cellular physiology can play a role. Different cell lines are utilized in different studies; most commonly transformed or tumour cells. As such, these cells are not necessarily representative of the average eukaryotic cell. Instantaneous toxicity has not been reported; rather cytotoxicity has been a function of time and concentration.\textsuperscript{41,43} QDs can be used to label cells.
early in the developmental stage without later detrimental effects. Slotkin et al. introduced commercial phospholipid CdSe/ZnS QDs into developing mouse neural stem and progenitor cells (NSPC) at the two-cell embryo stage via electroporation. The cells continued to develop, migrate and differentiate normally throughout the development process. The resistance of QDs to chemical degradation and photobleaching allowed their use for imaging of cells in utero.

1.5.1 Mechanisms for delivery

There exist three main mechanisms for intracellular delivery: active, passive and facilitated delivery. The methods are largely taken from established oligonucleotide delivery techniques. Similarities in charge and size between QDs and oligonucleotides have resulted in the proposed practicality of these techniques. The focus here will be on facilitated delivery mechanisms as this was employed in this project.
1.5.1.1 Passive Delivery

Passive delivery methods rely on membrane-QD interactions that are largely electrostatic in nature. Other than rendering the QDs hydrophilic, there is no additional functionalization to aid cellular internalization. Non-specific endocytosis has been observed using DHLA-capped CdSe/ZnS QDs at concentrations of 400-600 nm after 2-3 hours.\(^\text{46}\) While no major cytotoxicity issues were observed over several days in culture, QDs remained sequestered within endosomal compartments. Poly(ethylene glycol) (PEG) coated QDs (an inert coating that minimizes cellular uptake through endocytosis) when subjected to passive delivery exhibited co-localization only at the extracellular membrane.\(^\text{33}\) Intracellular distribution was observed to be dependent on size. A variety of CdTe QDs, ranging from 2-6 nm in diameter were solubilized using thioglycolic acid and introduced to a variety of cells including macrophages, phagocytic, epithelial and endothelial cells via passive delivery. Smaller QDs (~2 nm) were located in the nucleus while larger QDs (~6 nm) remained in the cytoplasm.\(^\text{47}\) The main advantage of passive delivery is its simplicity.
QDs only need to be rendered water soluble and then can be introduced to a cell culture, allowing for large numbers of cells to be exposed. Unfortunately, this method does not address specific cell types and its endosomal entrapment means QDs delivered in this manner were not capable of targeting specific organelles nor can they offer cytoplasm probing capabilities. Furthermore, this technique requires high concentrations of cells and long incubation times, and the latter can could contribute to cytotoxicity.

1.5.1.2 Active Delivery

Two main techniques exist for active delivery, both of which require direct physical manipulation of the cell: electroporation and microinjection. Electroporation has been used to transflect DNA. An electrical pulse is sent through the cell to temporarily permeabilize the phospholipid bilayer of the plasma membrane. Although QDs have been internalized using this method, the formation of large aggregates, up to 500 nm in diameter was observed within the cytoplasm. Similar QDs introduced using facilitated delivery techniques did not show aggregation suggesting that the electrical pulse/field affects the resulting intracellular QD dispersion. Electroporation has advantages in bypassing the endocytic pathway while maintaining the ability to introduce QDs to a large number of cells. However, the strong electric pulse required for permeabilization can result in cell mortality.

Microinjection delivers tiny volumes (femtoliter scale) directly into the cytoplasm of individual cells, avoiding endosomal trapping. Cells have been placed under a microscope and a fine tipped glass microcapillary has been used to introduce QDs into the cells. QDs were observed throughout the cytoplasm. Functionalizing QDs with targeting peptides such as nuclear localization sequence (NLS) and mitochondria localization sequence (MLS) resulted in corresponding co-localization within 30 minutes and was observed to increase over 18-24 hrs. This timescale is consistent with the cytoplasmic transport of larger complexes such as adeno-associated viruses. The functionalized QDs were PEG coated and were generally ~25 nm in size. This technique has low cell mortality rates, but is very limited in the number of cells that can be treated, and is costly and time consuming. Each individual cell needs to selected, visualized and then injected. There also exists an issue concerning cell morphology such as membrane thickness.
1.5.1.3 Facilitated Delivery

Intracellular delivery via facilitated delivery requires decorating the surface of the QD with specific molecules such as proteins or peptides or the encapsulation of QDs in endocytic delivery polymers to achieve endocytosis. The specific functionalization dictates the initial interaction between QD-bioconjugate and cellular membrane. It is important to note that if bioconjugation is not efficient, the ability of QD-bioconjugates to penetrate cells may be influenced by the unconjugated surface coatings of the QD.

Endocytosis is a process by which cells uptake nutrients, downregulate growth factor receptors and regulate signaling circuitry. There are many different types of endocytosis but all feature the formation of intracellular vesicles initiated by the invagination of the plasma membrane. Phagocytosis or “cell eating” takes up particles that are larger than 0.5 µm. Pinocytosis or “cell drinking” internalizes fluids surrounding the cell. For QD delivery, where QDs were often first introduced to the cell medium, pinocytosis is the proposed pathway. There are also endocytic pathways that are mediated by specific molecules. The best defined pathway is clathrin-mediated endocytosis. Endocytic vesicles containing the cargo for delivery are small, generally less than 100 nm and will fuse with early endosomes.

Peptide sequences (MW<3 kD) can be used to initiate interactions with the cellular membrane either through specific receptors or by electrostatic interaction. Many QD-peptide-bioconjugates have been reported to remain trapped in endolysosomal compartments. Commonly used peptides include: TAT, derived from the HIV-1 virus which is arginine and/or lysine rich allowing for a positively charged QD-bioconjugate to interact with negatively charged receptors on the cell surface. Pep-1 is a synthetic protein commonly used as a non-covalent carrier of peptides, proteins and nucleic acids into cells. It features a lysine rich region for solubility while its hydrophobic core interacts with the cell membrane. The tripeptide arginine-glycine-asparatate (RGD) interacts specifically with heterodimeric cell surface receptors known as integrins for intracellular delivery.

Protein mediated delivery requires that the entire protein be present on the surface of the QD. This model involves proteins recognizing and binding to specific markers or receptors on the surface of cells. A common protein is epidermal growth factor (EGF) which binds to a specific targeted membrane receptor which encourages endocytosis upon binding. QDs introduced
using EGF have been used in labeling studies, tracking the internalized receptor dynamics over time. EGF expression is upregulated in many different tumour types and therefore is considered a therapeutic target.\textsuperscript{41} Transferrin is another protein which actively undergoes endocytosis after binding to the transferrin receptor. It is a serum glycoprotein which ferries iron across the cellular membrane.\textsuperscript{39,41} The use of transferrin has been used to deliver QDs to different strains of bacteria.\textsuperscript{48} Antibodies, can also be used to guide QD-bioconjugates towards specific cell surface proteins that are actively endocytosed.\textsuperscript{41} Antibodies are of particular use for targeting specific cancer cells that often overexpress specific membrane markers.

Polymer based delivery techniques use amphiphilic groups to surround solubilized QDs and interact with the lipid cellular membranes to encourage endocytosis. Polymers also provide the possibility of additional functional groups for further chemical modification. Commercial cationic liposomal reagents such as Lipofectamine 2000 and Cellfectin ® were initially used for transfection of plasmid DNA into mammalian or insect cells.\textsuperscript{33} They have since been used for delivery of QDs, and initiate the process of assembly with negatively charged QDs. In a study comparing active and facilitated delivery techniques, cationic liposomes exhibited the highest delivery of QDs into live cells.\textsuperscript{33} Transmission electron microscopy (TEM) visualized a single QD-PEG-liposome on the range of 200-500 nm in diameter and coated with 20-40 QDs.

PEG-QDs were observed after transfection in the cytoplasm of HeLa cells.\textsuperscript{33} QDs were observed to have escaped the endosome and accessed the cytoplasm. This was determined by complexing green emitting QDs with transfection agents while red emitting QDs were coated by adsorption with EGF to encourage endocytosis. Green QDs were not observed to be co-localized with endolysosomal compartments as determined by a marker for acidic organelles (LysoSensor Blue).\textsuperscript{33} Red QDs on the other hand were observed almost exclusively co-localized in endolysosomal compartments.\textsuperscript{33} Unfortunately, aggregation was observed after endosomal escape.

Other liposome based delivery methods have been designed to fuse with cellular membranes.\textsuperscript{41} To promote the release of QDs from endosomes, a specific endosome-disrupting polymer polyethyleneimine (PEI) has been introduced as a branched copolymer for delivery of QDs. PEI can act as a “proton sponge” due to the large number of amines present to cause the disruption of
acidic endosomes and release QDs into the cytoplasm. It is important to note that PEI requires incorporation with other ligands as it is known to have cytotoxic effects.

Small molecules have also been used as an intracellular delivery method. CdTe/CdS QDs have been rendered soluble with MPA and then were non-covalently complexed with glucose to monitor the kinetics of glucose uptake activity in live yeast cells. QD-MPA-glucose bioconjugates were observed in the cytoplasm within 10 minutes of incubation. This result suggests that sugar based complexes can be used with QDs for transport using integral membrane transport. This is despite the fact that glucose molecules are several orders of magnitude smaller than QDs.

### 1.5.2 Endosomal Escape

Endosomal escape is an important step after intracellular delivery for QD-bioconjugates if they are to serve as bioprobes. Early endosomes have near neutral pH while late endosomes/lysosomes have more acidic pHs. Endosomes have been reported to be highly acidic with pHs of 4.0-5.5 which can degrade QD-bioconjugates over time depending on the functionalization. Nabiev et al. used thioglycolic acids to render their CdTe QDs soluble and observed endosomal escape from a variety of cells. It was reported that the reversible protonation of thioglycolic acid aided in the endosomal escape. The proton sponge effect has also been suggested for endosomal escape. As such, it is important to not only achieve endosomal escape but to have the QD-bioconjugates present in the cytoplasm as soon as possible to maintain their probing abilities. It has also been theorized that endosomal trapping may play a role in intracellular precipitation further hindering the probing abilities of QD-bioconjugates.

### 1.6 Drosophila melanogaster

#### 1.6.1 Drosophila as Model Organisms

*Drosophila melanogaster* are routinely used as model organisms due to their versatility, low risk or health hazards and well characterized genome. *Drosophila melanogaster* has been reported as a model of choice for developmental studies. Thanks to its small genome and sophisticated genetics it has established itself as the ideal intermediary between bacteria, yeasts and higher vertebrates. It is estimated that nearly 50% of the *Drosophila* gene products show similarities with predicted mammalian proteins. The DNA content of a *Drosophila* cell is
approximately 50x greater than that of *E. coli* and 30 times smaller than that of mammals.\textsuperscript{56} Mutations can be spontaneous or induced and will affect all aspects of its development and physiology making it of interest for genetics studies.\textsuperscript{56} mRNA and small interfering RNA (siRNA) expression has been well studied using a variety of assays and complementary techniques such as Western or southern blotting and chase screening. The interest of the work of this thesis was to study such expression *in vivo* using a QD based bioprobe.

### 1.6.2 Drosophila Cell Lines as Models

Optimal growth of *Drosophila* cells is at 22-25°C with doubling occurring about every 24 hours.\textsuperscript{56} The ease of use of *Drosophila* cell lines is one of their many advantages including i) no need for specialized incubators, ii) most culture media do not require a bicarbonate buffer requiring a controlled gas phase, iii) the cells tolerate large variations in pH and/or osmolality, iv) cells are usually grown in monolayers or as suspension cultures and do not require any enzymatic treatment for transfer and v) most lines can be kept frozen in liquid nitrogen over long periods of time. Rarely does a true and complete monolayer form and most cell lines have adapted to suspension cultures.

There are disadvantages compared to mammalian cell systems including difficulties in establishing new cell lines. Furthermore, the majority of currently available lines are derived from dissociated *Drosophila* embryos and as such their exact tissue origin is unknown. Every cell culture consists of dissociated and mixed cells from several hundred embryos of varying ages.\textsuperscript{56} *Drosophila* cells do not exhibit any “contact inhibition” and will exhibit a tendency to aggregate.

Transplanting isolated nuclei from permanent cell lines into fertilized eggs showed retention of the ability to guide a number of differentiation programs.\textsuperscript{56} This illustrated the conservation of genetic material. Furthermore, no obvious deviation from the fly genome was observed when cloning structures or chromosomal environments of any given gene from cultured cells.\textsuperscript{56}

The two main experimental models developed from *Drosophila* cell lines are heat shock response and the induction of proteins by hormonal treatment. The interest of the work in this thesis is the response to external stressors such as heat shock.
1.6.2.1 Kc Cells

The Kc cell line is one of the most widely used and studied *Drosophila* cell line. Kc167 cells were derived from disaggregated 8-12 h embryos and are clones of the original Kc line. It is a result of the F2 generation from a cross between *ebony* and *sepia* stocks. Kc167 cells have also been identified as hemocyte derivatives and are assumed to be a homogeneous female population. Kc167 cells were chosen due to having been reported as more efficient for transitory transfection.

1.7 Heat Shock Proteins

The heat shock response is universal and exhibited in organisms from bacteria to higher animals. The heat shock response consists of the induction of specific proteins after a brief exposure to elevated temperatures. The main heat shock proteins (hsp) have been conserved throughout evolution illustrating the importance of this phenomenon. A variety of other stressors such as treatment with heavy metals or alterations in oxygen flow can induce the same kind of response. The heat shock response is more of a reaction to external stressors and not just thermal perturbations. Due to the particularly intense response to heat shock displayed by *Drosophila*, *Drosophila* cell lines are one of the most regularly used experimental models for studying the mechanisms regulating gene expression in higher eukaryotes.

Heat shock response was first observed in *Drosophila* as the induction of new puffs on the polytene chromosomes of salivary glands. Similar responses were observed after exposure to various chemical or environmental stressors. A subset of the proteins expressed was observed more frequently after heat stressors and were named the heat shock proteins. Heat shock proteins were first reported by Cheney and Shearn to be present in imaginal disks of late third instar larvae in the absence of stress.

In *Drosophila melanogaster*, these proteins have been subdivided based on their molecular masses: the small heat shock proteins (shsp) with molecular weights of 22, 23, 26 and 27 kDa, the Hsp:Hsc70 family and hsp 83 with molecular weights of 70 and 83 kDa respectively. The hsp families also contain heat shock cognates (hsc) which function as molecular chaperones in aiding the folding and assembly of proteins under normal conditions.
Heat shock proteins are conserved proteins which act as molecular chaperones and are involved in a variety of cellular processes including protein folding or refolding, targeting and disposing of aggregated proteins, and translocation across membranes. They have also exhibited the ability to prevent intracellular damage induced by environmental stress during aging and neurodegenerative diseases. The overexpression of shsps can be beneficial to Drosophila as it has been shown that it can help extend lifespan and stress resistance.

1.7.1 Role of Heat Shock Proteins

Heat shock induces the rapid synthesis of new proteins while the rate of synthesis of most cellular proteins normally made at 25°C is greatly reduced. It results in the expression of specific mRNAs, some of which are preferentially translated into heat shock proteins. Preexisting polysomes in cells were observed to disappear quickly at 37°C while new, larger polysomes containing heat shock mRNAs appeared. There is evidence that proteins synthesized at 37°C are not involved in the repression of other active genes prior to heat shock. It was previously suggested that hsp have a role in the level of chromosomes during heat treatment. Experiments were performed where the synthesis of proteins was blocked before, during and after heat shock. This was done through the addition of protein synthesis inhibitors and tracing by the incorporation of radio-labeled elements. It was then observed whether the synthesis of specific proteins during heat shock was necessary for the restart of normal RNA synthesis at 25°C. It was of interest whether or not hsp were responsible for the repression of the rate of synthesis of many mRNA species normally present at 25°C. There seems to be a link between the restart of RNA synthesis and the migration of heat shock proteins to the cytoplasm, suggesting that hsp are required for re-initiation of mRNA synthesis after heat shock.

1.7.2 Heat Shock Factor

A heat stressor activates and increases the transcription of hsp genes and is regulated primarily by the heat shock transcription factor (HSF). The activity of HSF is controlled by a complex series of events induced upon exposure to stress. HSF is also required for oogenesis and early larval development under normal growth conditions.

HSF exists as a monomer distributed diffusely all over chromatin. During heat stress, it trimerizes in an activated form with high DNA-binding affinity. It then binds to distinct loci
including the major hsp loci. This binding event is coordinated by a conserved element known as the heat shock element (HSE). The binding event of HSF:HSE activates the transcription of various hsp genes resulting in their overexpression. A certain threshold of HSF must be bound to the HSEs before heat shock-induced expression of the hsp gene occurs. Full induction of shsps requires at least 3 HSEs localized over several hundred base pairs in front of the hsp genes. Within the cells, after heat shock it was reported that translation of heat shock mRNAs is favoured over normally synthesized mRNA. The optimal temperature for the induction of shsps is approximately 35°C.

1.7.3 Small Heat Shock Proteins

In cells, shsps act as molecular chaperones, preventing the aggregation of different protein substrates following exposure to stress. There are 4 main shsps which have been studied in detail in the literature: hsp22, hsp23, hsp26 and hsp27. All 4 share high sequence homology, are expressed following stresses in a similar and coordinated manner but have different developmental expression pattern and localization intracellularly. The expression of all 4 shsps after heat shock was observed in a coordinated pattern. In drosophila, all cells are capable of mounting heat shock responses at various stages of development. The function(s) of each of the shsps is controlled by different cell and developmental stage specific mechanisms and is a result of a combination of their expression, their cellular localization or their structure by post-translational modifications. Despite ongoing shsp research, there have been no reports of mutants in the shsp genes.

1.7.3.1 Location of shsp in Cells Before and After Heat Shock

Using classical cell lysis methods and electron microscope audioradiography of Kc cell sections, over 80% of hsp 22, 23, 26 and 27 were found in nuclei after heat shock bound to chromatin. Proteins in this study were labeled with 35S methionine. As the cells were allowed to return to 25°C, a large portion of shsps were observed to return to the cytoplasm. Furthermore, the normal pattern of protein synthesis was observed to take place coupled with a gradual decrease in the synthesis of hsp.

After an hour of heat shock, the cells were returned to 25°C and hsp were observed to be present in the cytoplasmic fractions of lysed cells. Heat shock proteins were synthesized even after heat
shock. It is not certain why expression continues after heat shock, it is believed that the stress remains for some time. Proteins which had been labeled between 2-3 hours into the recovery phase (ie: returning to 25˚C), were observed 8 hours later in the nuclei in lower proportions. This indicated that the cells had not yet recovered. After 10 hours, most of the hsp proteins which were initially observed in the nuclei fractions were now observed in the cytoplasmic fractions. A plateau was reached in terms of the amount of protein in the nuclei at around 5-6 hours. Maximum levels of hsp were observed in the nucleus after 2 hours followed by their progressive decrease. During recovery, RNA synthesis was observed to increase steadily until after a few hours it exhibited the same rates as a control group of cells left at 25˚C.

When a second heat shock was done on the cells which had already recovered, new hsp proteins were observed in the nuclei, nucleoli and chromatin and cytoplasm. It was also observed that hsp proteins synthesized during the initial heat shock were found to move back to the nuclei, migrating back to chromatin.

If kept at 37˚C, hsp proteins remain in the nuclei. Hsps labeled during a one hour 37˚C heat shock remain bound to chromatin when kept at 37˚C for several hours. After returning the cells to normal temperature (25˚C), the hsp proteins were observed to leave the chromatin.

### 1.7.3.2 Hsp23

Hsp23 is the small heat shock protein chosen as the target for the bioprobe design because it is one which is localized in parts of the cytosol uniformly. Furthermore, Vitek and Berger have reported that hsp23 mRNA is very stable in tissue culture cells.

Hsp23 and hsp27 exhibit cell specific expression patterns in the absence of stressors. They are also expressed under normal conditions.

### 1.8 Summary and Contributions

Applications featuring QDs as a basis for development of biosensor technology for nucleic acid detection has been developed by Algar. Algar has reported the use of QDs as FRET donors to simultaneously detect two different oligonucleotide sequences diagnostic for common pathogens: *Escherichia coli* and *Listeria monocytogenes*. His studies feature the immobilization of functionalized QDs onto optical fibers to serve as a diagnostic tool. His work
is the platform that has inspired the work presented in this thesis. Further inspiration has come from the literature where there is work that begins to take advantage of the spectroscopic properties of QDs for \textit{in vivo} applications. There is interest in using QDs not only for visualizing and labeling but also for diagnostics and monitoring purposes.

QDs have been presented in \textit{in vivo} applications for targeting and labeling specific cells. The chemistry involved with intracellular delivery features a wide range of cell penetrating peptides, proteins or aptamers targeting specific receptors.\cite{ENREF_12,ENREF_14,ENREF_24,ENREF_32,ENREF_34,ENREF_94,ENREF_95,ENREF_96,ENREF_97} Applications using QDs as scaffolds for the delivery of drugs has also been reported for advances in diagnostics as well as therapeutics. The work of this thesis focuses more on diagnostic applications in particular for genetic expression.

The ultimate objective of the work presented in this thesis is the construction of QD-bioprobes that can provide measurable signals corresponding to nucleic acid content within the cytoplasm of a cell. To the best of our knowledge, the presented work is the only one featuring the use of nucleic acid based QD-bioprobes for intracellular studies using \textit{Drosophila melanogaster} cells.

To achieve this goal, it was first necessary to design a QD-bioprobe that would show selectivity for a representative gene while generating a signal that was sufficient for detection. Signal transduction was of concern as the majority of FRET based hybridization detection methods feature the use of labeled targets; a technique not practical for intracellular environments. The proposed system featured a competitive displacement assay. A QD was conjugated with a single stranded oligonucleotide probe. A partially complementary partner (target) labeled with a molecular dye provided a labeled hybrid. The molecular dye was chosen such that it would form a FRET pair with the QD. FRET sensitized emission of the fluorophore was observed with high efficiencies. Upon introduction of the unlabeled target strand, FRET sensitized emission was observed to decrease concurrently with an increase in QD PL emission confirming that displacement of the labeled target strand had occurred.

There were concerns as to whether or not the initial FRET sensitized emission was a result of hybridization or simply adsorption to the surface of the QD. To address such a possibility, a non-complementary probe was introduced. It was determined that the presence of a non-complementary probe did not compromise selectivity for the target strand and was effective in reducing undesired non-specific adsorption. Another aspect of concern was the possibility of
slow kinetics of reaction involved in a competitive displacement assay. Engineering of the
stability of the QD-probe+target system was based on the design of the sequence of the labeled
target to be pre-hybridized with the QD-probe bioconjugate, and is described in Chapter 3. A
variety of destabilizing properties such as mismatched base pairs and sequence length was
studied to develop an assay system that promoted displacement.

Once a QD-bioprobe system was developed that showed promising results in vitro, protocols
were developed for introduction into Drosophila melanogaster Kc cells. Intracellular delivery
was achieved via transfection. Preliminary assessment of the stability and function of the QD-
bioprobe in an intracellular environment was promising. FRET sensitized emission was retained
over extended periods of time. The efficacy of the QD-bioprobe in an intracellular environment
was not well characterized and remains subject to interpretation. These results are discussed in
Chapter 4.

The results presented in this thesis provide a platform for future work using QDs in diagnostic
and monitoring schemes in intracellular environments. Outlines of future directions using the
developed system conclude this thesis.


(39) McGrath, N.; Barroso, M. *Journal of Biomedical Optics* **2008**, *13*.


(49) Yezhelyev, M. V.; Qi, L. F.; O'Regan, R. M.; Nie, S.; Gao, X. H. *Journal of the American Chemical Society* **2008**, *130*, 9006.


Abstract

Quantum dots (QDs) were used as the scaffold for a nucleic acid bioprobe for the detection of a sequence selective for hsp23, a heat shock protein expressed by Drosophila melanogaster cells upon heat stress. QDs were used as fluorescence resonance energy transfer (FRET) donors in previous nucleic acid hybridization detection schemes. Bidentate thiol chemistry was used to attach oligonucleotides onto green 525 CdSe/ZnS QDs via self-assembly to yield stable QD-DNA conjugates. Upon hybridization with Cy3 labeled target strands, FRET sensitized emission of Cy3 was visualized with approximately 50% FRET efficiency. Increases in quantum yield and photoluminescence (PL) lifetimes were observed after the attachment of oligonucleotide probes. Förster distances and energy transfer efficiencies were determined. The dynamic range and limit of detection were dependent on the ratio of QD:oligonucleotide probe. The optimal ratio required a balance between space on the surface of the QD available for conjugation while minimizing the potential for non-specific adsorption. The proposed QD-DNA conjugate featured the presence of non-complementary sequences to block adsorption sites on the surface of the QD. It was observed that the presence of non-complementary probes did not affect selectivity and reduced non-specific adsorption. The optimal ratios of QD to hsp23 probe to a non-complementary oligonucleotide were determined to be 1:4:0 and 1:4:1. Saturating the QD:DNA probe was observed at a ratio of 1:2 of probe to target. The ratio of QD:probe also plays a role in the speed of hybridization. Doubling the amount of probe available resulted in a decrease in rate by a factor of 2.

2.1 Introduction

Bioconjugation of nucleic acids to semiconductor nanocrystals known as quantum dots (QDs) for use in sensor development is just one of the many applications of QDs that have been reported recently.\textsuperscript{1-8} There is wide interest in the use of QDs in place of conventional organic dyes due to extensively reported photophysical properties including greater quantum yield, resistance to
photobleaching and chemical degradation, narrow size-tunable emission, broad absorption and overall photostability.\textsuperscript{9} This allows for labeling and tracking over extended periods of time which is particularly beneficial for cellular imaging and long term studies. Biological applications of QDs involving nucleic acids include the use of QDs as a scaffold for drug delivery and imaging\textsuperscript{10-13} and \textit{in situ} hybridization studies.\textsuperscript{14-16} There has also been work done on the use of QDs for monitoring the delivery of siRNA.\textsuperscript{14}

The use of QDs as energy donors for Fluorescence Resonance Energy Transfer (FRET) is well established.\textsuperscript{7,8,15} However, many reported applications feature the use of labeled targets. For \textit{in vivo} studies, labeling a target nucleic acid sequence with an organic dye is not possible. It is difficult to label naturally synthesized nucleic acids. The use of a sandwich assay features two individual strands labeled with corresponding FRET pair dyes. Hybridization with the target nucleic acid sequence results in the close proximity required for FRET.\textsuperscript{16} This transduction scheme has not been successfully reported, likely due to the issue of degradation by nucleases of single stranded oligonucleotides. Another method labeled synthesized proteins using $^{35}$S methionine but required cell lysis prior to analysis.\textsuperscript{17} The proposed QD based bioprobe used FRET as a transduction method that can be performed in live cells.

The proposed bioprobe featured a stable QD-DNA conjugate pre-hybridized to a target strand labeled with Cyanine 3 (Cy3). The resulting QD-DNA+target construct was stable in aqueous solution over extended periods (several months). The probe oligonucleotide was complementary to a target sequence within the heat shock protein 23 (hsp23) gene mRNA sequence upregulated by \textit{Drosophila melanogaster} after undergoing an external stressor such as heat shock. This scheme can further current knowledge on gene expression and cellular response pathways. Heat shock genes are well characterized and are often used for genetic studies.

Optimization of the QD-DNA conjugate was performed by determining loading capacity and minimizing non-specific adsorption which was a well-known issue with MPA QDs.\textsuperscript{18} An optimal ratio of QD to probe was determined to ensure balance between sufficient space for hybridization while blocking adsorption sites was determined. The use of non-complementary probe sequences to block adsorption sites was also investigated.
2.2 Reagents and Instruments

CdSe/ZnS core/shell semiconductor nanocrystals quantum dots (QDs) in toluene with an emission maximum of 524 nm were from Evident Technologies (Troy, NY, USA). CdSe/ZnS core/shell QDs were also synthesized in-house. These QDs had an emission maximum of 536 nm and were kept in chloroform. 98% 3-mercaptopropionic acid (MPA), 99% N,N-diisopropylethylamine (DIPEA) and Tris(2-carboxyethyl)phosphine (TCEP) were from Sigma Aldrich (Oakville, ON, Canada) and used without further purification. Chloroform was from Caledon Laboratories (Georgetown, ON, Canada) and used as received.

Water was deionized and purified using a Milli-Q cartridge purification system (Millipore Corp, Mississauga, ON, Canada). Buffer solutions were prepared using autoclaved deionized water (TB buffer 50 mM, pH 7.6; Borate buffer 50 mM, pH 8.4 and 9.5). Buffers with increased ionic strength were prepared by the addition of NaCl to a final concentration of 25 mM.

Modified and unmodified oligonucleotides were from Integrated DNA Technologies (Coralville, IA, USA) and were HPLC purified by the manufacturer. Oligonucleotides were dissolved using autoclaved MilliQ water and filtered through a 0.2 µm syringe filter prior to use.

Ultraviolet-visible absorption spectra were collected using a Hewlett Packard 8452A Diode Array Spectrophotometer (Hewlett Packard Corporation, Palo Alto, CA, USA). Steady state fluorescence spectra of solutions were collected using a QuantaMaster PTI spectrofluorimeter and analyzed using Felix software (Photon Technology International, Lawrenceville, NJ, USA). Photoluminescence lifetime measurements were done using a time correlated single photon counter (constructed in-house). A GL-302 dye laser (PTI) using DPS (in dioxane) which was excited with a GL03300 nitrogen laser (PTI). Data was analyzed using TMaster software (Photon Technology International, Lawrenceville, NJ, USA).

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp23 probe</td>
<td>5’-(-/5DTPA/ TTT TAA TTG CTT CAA GAT GAT TCT C -3’</td>
</tr>
</tbody>
</table>
uidA sequence 5’ -/5DTPA/ CTT ACT TCC ATG ATT TCT TTA ACT - 3’

Fully complementary target (18bp-FC)

2.3 Experimental

2.3.1 Ligand exchange by heating

Organic QDs were diluted in chloroform and an excess (1:50 000) of mercaptopropionic acid (MPA) was added along with enough N,N-diisopropylethylamine to render the solution basic. The solution was refluxed under Ar for 5-8h at 60-70˚C. The precipitate and supernatant were then centrifuged at 8000 rpm for 4 minutes to achieve a pellet. The pellet was washed with chloroform by mixing and centrifugation three times to remove excess ligands. The supernatant was removed each time. Finally, the precipitate was re-dissolved in a small amount of basic borate buffer. Ethanol was added until the solution became turbid. The mixture was centrifuged once more and the supernatant removed. This step was repeated once more before the QDs were finally dissolved in the desired amount of deionized water. The concentration of the QDs was determined by absorption spectroscopy using the first absorption peak at 504 nm.

2.3.2 Ligand Exchange by Base Washing

An excess of ligand (QD:MPA 1:50 000) was diluted with dichloromethane and an excess of tetramethyl ammonium hydroxide pentahydrate was added to render the solution basic. The solution was shaken vigorously and then allowed to equilibrate over 1-2 hrs. Two layers were observed and the desired de-protonated ligand was present in the organic layer. The organic layer was removed and added to a solution of organic QDs. The solution was shaken vigorously and given 12-24 hrs to equilibrate. The coated QDs formed small aqueous pellets which were coloured and easily distinguished from the organic layer. The solution was observed under ultraviolet (UV) light to ensure a minimal amount of QDs was left in the organic layer. The organic layer was then removed. The QDs were re-dissolved in a minimal amount of basic borate buffer containing salt. Ethanol was added until the solution became turbid. The mixture was centrifuged and the supernatant removed. This step was repeated once more before the QDs were
then dissolved in the desired amount of deionized water. The concentration of the QDs was determined by absorption spectroscopy using the first absorption peak at 504 nm.

### 2.3.3 Preparation of QD-DNA Bioconjugates

QD-DNA bioconjugates were achieved by preparing a dispersion containing MPA coated QDs, the desired equivalent of dithiol modified oligonucleotide probe and TCEP in at least 1000 fold excess. The solutions were diluted using the appropriate buffer, mixed using a vortex mixer and then allowed to incubate at room temperature for 5 hours to overnight. TCEP was added as it selectively reduces dithiol bonds. TCEP solutions were kept at 4°C for no longer than a week. In the case of mixed probe conjugates, fully complementary hsp23 probe was always added prior to the non-complementary uidA sequence to maintain consistency.

Stock solutions of QD-DNA bioconjugates were stored at 4°C until further use. Stock solutions had concentrations between 500 nM to 2.5 µM. Dilutions from stock solutions were done to achieve the desired concentrations using the desired buffers. Experimental conditions featured solutions containing a final concentration of between 100-250 nM QD-bioconjugates.

### 2.3.4 Loading Capacity Experiments

QD-nDNA bioconjugates with \( n = 1, 2, 4, 6, 8 \) or 10 equivalents of dithiol modified oligonucleotide probe using an excess of TCEP were prepared as previously described. QD-DNA bioconjugates were either exclusively hsp23 or uidA. QD-nDNA bioconjugates with exclusively non-complementary uidA sequence served as a control experiment. A Cy3 labeled target nucleic acid sequence fully complementary to the hsp23 probe was added in excess to achieve a final concentration of 1 µM. This concentration was approximately 2-5 equivalents of labeled target to probe oligonucleotide. The solutions were mixed quickly by vortexing before incubating at room temperature in the dark for 5 hours to overnight.

Fluorescence spectra were collected with excitation at 405 nm and emission was collected from 480 nm to 650 nm, 24 to 30 hrs after the addition of target. Assessment of the optimal QD: probe ratio was determined by maximal FRET efficiency.
2.3.5 Dynamic Range Estimation

The optimal amount of target added to QD-DNA bioconjugates to achieve a maximum FRET signal was calculated based on a ratio of moles of target to moles of hsp23 probe available per QD. Ratios measured were 0, 0.5, 1.0, 1.5, 2.0 and 2.5. Solutions were prepared using 50-100 nM of QD-DNA bioconjugate using 50 mM Tris-Borate buffer, pH 7.6. Solutions were mixed quickly by vortexing and allowed to incubate at room temperature for a minimum of 12 hours to achieve complete hybridization. The molar ratio of target to probe was plotted against the FRET ratio of FRET-sensitized Cy3 fluorescence emission to QD fluorescence (Eq. 2.8).

2.3.6 Kinetics Experiments

Kinetics experiments were done by preparing 100 nM solutions of MPA QDs or QD-DNA conjugates at the desired QD: probe ratios in 50 mM TB buffer with or without an additional 25 mM NaCl. The resulting amounts of probe available in solution were 0.0, 1.0, 2.0, 3.0 or 4.0x10^{-11} moles for 1:0, 1:1, 1:2, 1:3 and 1:4 QD-DNA conjugates respectively. Non-specific adsorption was also tested by using a 1:1 QD-DNA conjugate prepared with uidA sequence. An excess (1.0 x10^{-10} moles) of Cy3 labeled target nucleic acid strand was added. Based on the dynamic range experiments, it had been determined that 2 equivalents of labeled target was sufficient to saturate the QD-DNA conjugate. The solution was mixed quickly by vortexing and spectra were collected with excitation at 405 nm for 4-10 hours. Spectra were collected at 1 minute increments for the first 15 minutes then at five minute increments for the remaining first hour, and then at 10 minute increments. FRET ratios were calculated as the ratio of fluorescence emission of acceptor (Cy3) and fluorescence emission of the donor (QD) (Eq. 2.8).

2.3.7 Calculating Kinetic Parameters, Quantum Yield, Lifetimes, Förster Distances, FRET Efficiency and FRET Ratios

The kinetic curves obtained were fit to the exponential function shown in Eq. 2.1.

\[ y = A(1 - e^{-bx}) \]  

where \(y\) corresponds to the FRET ratio between acceptor (Cy3) emission and donor (QD) emission as a function of time, \(x\) is the time parameter in minutes and \(A\) is a constant. The
kinetic parameter $b$ was of interest as it describes the rate of hybridization or adsorption. Best fit curves were obtained using OriginPro 8.0 scientific graphing and analysis software.

Quantum yield values for QDs were determined as a ratio relative to fluorescein dye in pH 9.5 sodium borate buffer. Fluorescein has a known a quantum yield of 0.93 under these conditions$^{20}$. The quantum yield, $\Phi$, of QDs was calculated using Eq. 2.2 which was simplified to Eq. 2.3 by applying the Beer-Lambert Law, such that

$$
\Phi = \Phi_{ref} \frac{\int F d\lambda}{A} \frac{A_{ref}}{\int F_{ref} d\lambda} \quad (2.2)
$$

$$
\Phi = \frac{\int F d\lambda}{\int F_{ref} d\lambda} = \frac{\varepsilon \Phi_c}{\varepsilon_{ref} \Phi_{ref} c_{ref}} \quad (2.3)
$$

the integrated emission, $Fd\lambda$, was corrected for different molar absorptivities, $\varepsilon$, at the wavelength of excitation and for concentration of the solution, $c$. Fluorescein was excited at 490 nm and QDs were excited at 406 nm. Fluorescence and absorbance were measured using the same aliquot of solution to eliminate the instrument factor parameter from the equation and minimize error. Furthermore, a consistent path length was used for both measurements. QD solutions for quantum yield determination ranged from 500 nM to 2 µM.

Photoluminescence lifetimes were measured using between 500 nM to 2 µM solutions of QD or QD-DNA bioconjugates. Decay curves were obtained using SPCM software (Version 8.50) and SPC-360 hardware (Becker and Hickl GmbH, Berlin, Germany) and analyzed using SPCImage (version 2.8.3.2921, Becker and Hickl). Decay curves were fit using 2 component exponential decay by minimizing the $\chi^2$ value.

Förster distances were calculated using Eq. 2.4 which describes the distance, $R_o$, as a function of several factors including the refractive index of the surrounding medium, $n$, the quantum yield, $\Phi_D$, of the donor (QDs), the relative orientation between donor emission and acceptor absorption
dipoles as described by the orientation factor, $\kappa^2$ and the degree of spectral resonance between the donor (QD) and acceptor (Cy3) as described by the spectral overlap integral $J$, which was determined using Eq. 2.5. $J$ is a function of the fluorescence intensity of the donor, $F_D$, and the molar absorptivity of the acceptor, $\varepsilon_A$ both as functions of wavelength. The Cy3 ultraviolet-visible (UV-vis) absorption spectrum was obtained using a 2 µM solution. QD donor fluorescence emission spectrum was obtained using 100 nM solutions of QD-DNA bioconjugates. The integrands were calculated using 2 nm increments and integrated numerically to determine the spectral overlap.

$$R_o^6 = 8.79 \times 10^{-28} \text{mol} \times (n^{-4}\kappa^2\Phi_D J) \quad (2.4)$$

$$J = \int \frac{F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda)d\lambda} \quad (2.5)$$

FRET efficiencies, $E$, were calculated using Eq. 2.6 and 2.7, which describe the relationship with lifetimes, $\tau$, or quantum yield, $\Phi$ in the presence of an acceptor, indicated by the subscript $DA$ or in the absence of an acceptor, indicated by the subscript $D$.

$$E = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{\Phi_{DA}}{\Phi_D} \quad (2.6)$$

$$E = \frac{nR_o^6}{nR_o^6 + r^6} \quad (2.7)$$

To calculate FRET ratios, the sample spectrum was first background subtracted. A FRET ratio, $R$, was then used to quantify the amount of FRET-sensitized Cy3 fluorescence using Eq. 2.8.

$$R = \left(\frac{\sum_{538}^{590} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)}\right)_{DA} - \left(\frac{\sum_{538}^{590} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)}\right)_{D} \quad \text{OR} \quad R = \left(\frac{\sum_{538}^{590} PL(\lambda)}{\sum_{538}^{558} PL(\lambda)}\right)_{DA} - \left(\frac{\sum_{558}^{560} PL(\lambda)}{\sum_{538}^{558} PL(\lambda)}\right)_D \quad (2.8)$$
The wavelengths chosen were of a spectral region of acceptor PL (Cy3; 560-590 nm or 560-580 nm for QD525 or QD536, respectively) and significant donor PL (QD; 528-558 nm or 538-558 nm for QD525 or QD536, respectively).

2.4 Results and Discussion

2.4.1 Preparation of QD-DNA Conjugates

The attachment of probe oligonucleotides was achieved based on the displacement of the MPA from the ZnS shell using dithiol modified oligonucleotides. The S⁻ coordinates strongly with the Zn²⁺ on the shell of the QD. It was important to use MPA as a ligand for water solubility and oligonucleotide probes with a dithiol modification. MPA is a mono-thiol ligand and thus attachment chemistry for MPA QDs was considered monodentate. Selectively reducing the thiols on modified oligonucleotides using TCEP allowed for a displacement reaction where dithiol modified oligonucleotides attach via bidentate chemistry, replacing the mono-dentate MPA ligand. Mercaptoacetic acid (MAA), although it is also monodentate was not chosen due to its shorter alkyl chain. It has been previously reported that bidentate complexes exhibited less density packing compared to monothiol ligands.²¹ They were also reported to be more stable due to the presence of two S⁻ coordinating with the shell.²¹ Displacement reactions were allowed to proceed for 6 hours to overnight to ensure the reaction was complete.

Due to the possibility of re-oxidation over time and to ensure that all available probes were reduced for maximum coordination, TCEP solutions that were used for QD-DNA conjugate preparation were kept at 4°C and were no older than 5 days. It was observed that using TCEP older than a week resulted in much lower FRET signals, corresponding to a reduction of the quantity of QD-DNA bioconjugates formed.

It was observed that adding TCEP to MPA coated QD solutions without the addition of probe oligonucleotides resulted in the eventual aggregation of QDs, generally over a period of months.
2.4.2 Loading Capacity

Assessment of the optimal QD:probe ratio was determined by evaluating the FRET ratio between Cy3 fluorescence emission maximum for increasing ratios of QD to probe. After allowing more than sufficient time for hybridization to take place (determined experimentally to be approximately 12 hours) it was observed that maximum FRET ratios were observed for QD:probe conjugates where $n = 0$. This result indicated that non-specific adsorption remained a significant factor with MPA coated QDs. In the absence of probes, there were no steric hindrance issues or orientation limitations to the adsorption of oligonucleotides.

![Spectra showing the FRET response after over 24 hrs and complete hybridization with 1 µM of Cy3 labeled target sequence and varying QD:probe ratios. QD:probe bioconjugate solutions were 100 nM. Spectra were normalized to the QD emission maximum.](image)

Figure 2.1. Spectra showing the FRET response after over 24 hrs and complete hybridization with 1 µM of Cy3 labeled target sequence and varying QD:probe ratios. QD:probe bioconjugate solutions were 100 nM. Spectra were normalized to the QD emission maximum.

Based on the strong FRET responses observed in Fig. 2.1 and the susceptibility of bare QDs (no probe attached) to non-specific adsorption, a similar experiment was done using an oligonucleotide sequence (uidA) that was the same length, featured the same modification on the 5’ end and was non-complementary to the target sequence. QD:DNA conjugates with a ratio of 1:1 still showed a strong FRET response (Fig. 2.2). This demonstrated that a Poisson distribution
of a single 25 base pair oligonucleotide on the surface of the QD in any orientation provided sufficient sites for non-specific adsorption to occur. A substantial decrease was observed by doubling the amount of probe. This suggested that in the presence of two oligonucleotides, sites available on the surface of the QD for adsorption were greatly diminished. The ratio of 1:4 QD:probe exhibited the greatest FRET efficiency in the fully complementary QD:probe system. The same ratio also showed a FRET response in the same system featuring uidA modified QDs. This was interpreted as FRET generated from non-specific adsorption.

![Graph showing FRET response](image)

**Figure 2.2.** Spectra showing FRET response over 24 hrs after the addition of 1 µM Cy3 labeled target with varying QD: nprobe bioconjugates using a non-complementary uidA probe sequence. QD:nprobe bioconjugate solutions were 100 nM. There was some shift observed in the emission maximum of QDs. Spectra were normalized to QD emission maximum.

The greatest FRET ratio for QD-DNA conjugates was observed for QD:nprobe ratio of 1:4 (Fig 2.1). This ratio also exhibited the greatest FRET efficiency: 65.1± 7.01%. However this value may be enhanced considering the FRET response observed for the same ratio in Fig 2.2.
In the presence of 6 or more non-complementary probes per QD, FRET response was insignificant suggesting that the loading capacity of these green QDs was between 4 to 5 oligonucleotides per QD. This is in accordance with Lee et al. who reported a maximum loading capacity of 5 25-mer DNA strands per PEG-ylated 530 nm QD. The proposed system used in this thesis also featured a 25 base pair oligonucleotide probe but used shorter MPA ligands to achieve solubility. QD-DNA conjugates containing hsp23 probe also showed significant decreases in FRET with QD:probe ratios greater than 1:6. This suggests that the presence of 6 oligonucleotide strands on a single QD provided too much steric hindrance for hybridization to take place and/or any non-specific adsorption of non-complementary strands to produce FRET. The steric hindrance can be attributed to unfavourable electrostatic interactions between negatively charged oligonucleotides and deprotonated carboxylic acids present on the surface of the QD. As a result, a balance must be established between the amount of probe and space available for hybridization to take place. To achieve this balance, mixed probe conjugates were developed with ratios of $x:y:z$ where $x$, $y$ and $z$ represent equivalents of QD, hsp23 and uidA probe respectively. The overall ratio of QD:probe was kept at either 1:4 or 1:5. FRET response observed in 1:1:4 and 1:1:3 QD-DNA conjugates were similar to those observed in 1:4 using uidA probe sequence (Fig 2.3). The FRET response of 1:1:3 was greater than 1:1:4; this suggested that there was still some non-specific interaction between labeled target oligonucleotides and the surface of the QD. If the signal observed was purely the result of successful hybridization, it was expected that the FRET response from QD:probe conjugates of the ratio 1:1:3 would be identical to that from QD:probe conjugates of the ratio 1:1:4. These results further corroborated the hypothesis that approximately 5 oligonucleotides per QD strikes a good balance between amount of probe and the space available on a QD surface.

It was important to take into account the Poisson distribution of QD:probe conjugation. It was possible that there are more or less probes present on the QD surface than the described ratio. However, the Poisson distribution states that a significant portion of the population of the species present in that ratio.
A minimal amount of non-complementary probe was necessary to retain specificity and adjust the available space for hybridization between the desired probe and a labeled target. Subsequent experiments compared QD:probes with the ratios 1:4:0, 1:4:1 and 1:5:0.

**Figure 2.3.** Spectra showing response over 24 hours after the addition of a 1µM Cy3 labeled target. QD:probe ratios are written in the form of QD:hsp23:uidA in terms of molar ratios. QD:probe conjugate solutions were 100 nM. Spectra were normalized to QD emission maximum.

**Note:** The majority of QD-DNA conjugates used for hybridization experiments with Cy3 labeled target were not purified after self assembly. This was in an attempt to simulate the matrix that is the intracellular fluid or cytoplasm that the bioprobes were ultimately destined for. It is expected that in vivo, large amounts of other proteins, oligonucleotides and general intracellular debris would be present. By not purifying the samples, nucleotides are present in bulk solution. As a result, the FRET efficiencies and ratios are likely to be significantly less than in an ideal bulk hybridization assay. This was because in the unpurified samples, bulk solution oligonucleotides strands may hybridize with target strands. As FRET is a distance dependent phenomenon, not all the hybridized dsDNA probe-target complexes will exhibit fluorescence.
2.4.3 Dynamic Range

![Plot of target to probe molar ratios (based on corresponding QD:hsp23:uidA conjugate ratios) versus FRET ratios to illustrate the dynamic range QD:DNA conjugates.](image)

**Figure 2.4.** Plot of target to probe molar ratios (based on corresponding QD:hsp23:uidA conjugate ratios) versus FRET ratios to illustrate the dynamic range QD:DNA conjugates.

It was observed from Fig. 2.4 that 1:5:0 QD-DNA conjugates exhibited greater FRET ratios than 1:4:1 and 1:4:0. This was expected as 1:5:0 had an additional hsp23 probe available for hybridization.

Saturation of the QD:probe conjugates occurred at approximately 1.75 equivalents of target to probe. The response of the QD-DNA bioconjugates was fairly linear over a range of 0.5 to 1.5 equivalents of target to probe. This indicated a small dynamic range as an order of magnitude is usually expected for bioprobes or biosensors. QD:hsp23probe:uidA conjugates of the ratios 1:4:1 and 1:4:0 exhibited similar responses. It was expected that 1:4:1 and 1:5:0 would have similar responses based on the fact that their overall ratio of QD:probe was 1:5. However, 1:4:1 and 1:4:0 QD-conjugates were decorated with the same amount of hsp23 probe. The presence of a non-complementary probe did not hinder hybridization; this indicated that the system retained
selectivity. Furthermore it provided steric hindrance by blocking sites on the surface of the QD, resulting in enhanced sensitivity. The presence of non-complementary probes also lessened non-specific adsorption by providing steric hindrance in addition to increasing the charge density on the surface of the QD.

### 2.4.4 Kinetics

![Graph](image)

**Figure 2.5.** FRET ratios as a function of time for solutions of 100 nM bare MPA coated QDs (red dots) and QD-DNA conjugates using hsp23 probe (black squares) or non-complementary uid A sequence (blue triangles) after the addition of 1µM of Cy3 labeled target. Experiments were done in 50 mM TB buffer with an additional 25 mM NaCl. FRET ratios were calculated using Eq. 2.8.

**Table 2.2.** Tabulated kinetic parameter values for kinetic experiments with varying ratios of QD:hsp23 probe. Higher ionic strength conditions were achieved using buffers of the same pH with an addition of 25 mM NaCl.

<table>
<thead>
<tr>
<th>QD-DNA conjugate</th>
<th>$b$ value (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non complementary</strong></td>
<td></td>
</tr>
<tr>
<td>1:1 – uidA oligonucleotide sequence</td>
<td>$8.39 \times 10^{-4} \pm 2.58 \times 10^{-4}$</td>
</tr>
<tr>
<td><strong>Complementary</strong></td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
<td>Condition</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>1:0</td>
<td>salt</td>
</tr>
<tr>
<td>1:1</td>
<td>no salt</td>
</tr>
<tr>
<td>1:1</td>
<td>salt</td>
</tr>
<tr>
<td>1:2</td>
<td>no salt</td>
</tr>
<tr>
<td>1:2</td>
<td>salt</td>
</tr>
<tr>
<td>1:3</td>
<td>no salt</td>
</tr>
<tr>
<td>1:3</td>
<td>salt</td>
</tr>
<tr>
<td>1:4</td>
<td>no salt</td>
</tr>
<tr>
<td>1:4</td>
<td>salt</td>
</tr>
</tbody>
</table>

It was known that pH has an effect on adsorption of oligonucleotides to the surface of QDs.\textsuperscript{21,26} Higher pH values resulted in less adsorption. Normal intracellular fluid in \textit{Drosophila} cells is slightly acidic. Experiments were done using tris borate buffer at pH 7.6 to ensure the solubility of QDs. For experiments done using solutions of increased ionic strength, NaCl was added to a final concentration of 25 mM NaCl. Tris Borate buffer was chosen as it has been previously reported to support faster hybridization kinetics.\textsuperscript{23}

The pKa for MPA has been reported to be 4.3\textsuperscript{24}; however it has also been reported that when coated to nanoparticles or in thin films, the pKa of carboxylic acids can increase by several units. MPA coated QDs have been reported to have a pKa or approximately 7.8.\textsuperscript{18} Experiments were done in TB buffer at pH 7.6. At this pH, the surface carboxylates were present in both ionized and non ionized forms. As a result, non-specific adsorption was still an issue.

Ionization was expected to inhibit adsorption due to decreased hydrogen bonding interactions; the -OH group can act as a donor or acceptor while the carbonyl group acts as an acceptor. Ionized carboxylates (COO-) cannot act as donors. Furthermore, ionization would increase the
negative charge density at the surface of the QD and increase electrostatic repulsion forces with negatively charged oligonucleotides.

Bare MPA coated QDs showed continual increase in FRET response over 5 hours. Within several minutes, the FRET ratio was observed to be substantially greater than that observed in 1:1 QD:probe conjugates (Fig 2.5). Also, the extremely low value of the kinetic parameter, $b$, $5.35 \times 10^{-4} \pm 3.06 \times 10^{-4}$ min$^{-1}$ indicated that initial adsorption occurred very quickly and was able to generate strong FRET responses. The continual increase in FRET response observed by the bare QD system suggested that there was continual interaction between the surface of the QD and the labeled oligonucleotide strands. A possible model features the oligonucleotide lying flat on the surface of the QD or wrapped around the QD.$^{18,23}$ Over time, in the presence of excess amounts of target, the rearrangement of the orientation of the strands to extend from the surface may allow for more acceptors (Cy3 molecules) to be within the Förster distance for FRET. This would account for the increasing FRET response observed over time for bare QDs. As the pH of the system was close to the recently reported pKa of MPA ligands on surfaces$^{18}$, there was a population mixture of both ionized and non ionized MPA ligands. As a result, the system was still capable of supporting non-specific adsorption.

Hybridization occurs on a slower timescale compared to adsorption as expected as observed in Fig. 2.5. Target strands must satisfy two conditions for hybridization: i) proximal to the probe and ii) proper orientation in order for hybridization to occur. The continual increase in FRET response observed for bare MPA QDs indicated that the nucleotide-surface interaction was dynamic. This was reasonable considering the negatively charged nature of DNA strands and the population mixture of deprotonated or protonated MPA ligands. The plateaus observed in QD-DNA conjugates indicated that nucleotide-nucleotide interactions (aka Watson Crick base pairing) were more thermodynamically favoured and were much more stable.
The non-complementary system yielded the lowest FRET response (Fig. 2.5). This confirmed that the system was specific towards the hsp23 target strand. The non-complementary system also exhibited FRET within the first minutes of initiating interactions confirming non-specific adsorption.

Figure 2.6. FRET response as a function of time for solutions of 100 nM QD-DNA conjugates at varying QD:probe ratios (i-1:2, ii-1:3 and iii-1:4) after the addition of 1 µM Cy3 labeled target. Target oligonucleotide was always present in at least twice the amount of probe; as such all experiments were done under saturating conditions. Kinetic traces evaluated the presence of 25 mM NaCl (black squares) or no salt (red dots) in solution. FRET ratios were calculated using photoluminescence signals at emission maxima of Cy3 (566 nm) and QDs (524 nm).

Increasing the number of probes resulted in greater FRET ratios as it provided for more acceptors to be located around the QD donor. The presence of additional probes did not have a significant difference in the time required to achieve complete hybridization (c.a. 450-500 min). The FRET ratio observed within the initial minutes increased as the number of probes was increased. The kinetic parameter also increased as the number of probes was increased (Table 2.2). These results suggested adsorption occurred concurrently with hybridization. This was
consistent with previous observations that adsorption of oligonucleotides to interfaces occurred prior to the selective process of base pairing.\textsuperscript{23}

Doubling the amount of probe from 1 to 2 did not result in a large change in $b$ 0.0038 and 0.0033 min\textsuperscript{-1}, respectively. The fast kinetics observed were associated with non-specific adsorption. A ratio of 2 probes per QD was not saturating conditions; therefore there would still be a large surface available for adsorption on the surface of the QD. Doubling the probe from 2 to 4 resulted in an increase of 23 times in the kinetic parameter 0.078 compared to 0.0038 min\textsuperscript{-1}. This further corroborated that initial FRET was the result of adsorption. Hybridization requires a longer timescale as it is a more selective process requiring the appropriate physical and spatial configuration. Adsorption, on the other hand, has no constraints. Increasing the amount of probe also served to limit space available for adsorption. This would allow hybridization kinetics to dominate the kinetic parameter.

The effect of ionic strength was also investigated. It has been previously reported that for QD-DNA systems, pH played a greater role in hybridization kinetics but ionic strength was also a factor.\textsuperscript{23} It was observed in Fig 2.6 that in solutions with no additional salt, FRET ratios within the initial minutes were similar regardless of the amount of probe available. Also in the absence of salt, FRET intensity was lower and achieved equilibrium faster. These results suggested that the presence of additional ions may increase the rate of adsorption. The presence of additional ions contribute to charge screening effects, may also lower electrostatic repulsion allowing for adsorption and subsequently hybridization to occur. The kinetic parameter $b$ was approximately 1 to 2 orders of magnitude greater for QD-DNA conjugates in the absence of salt compared to those in solutions with adjusted with NaCl to a final concentration of 25 mM.

2.4.5 Optical Properties of QD-DNA Conjugates

Table 2.3. Measured quantum yield and lifetime values for MPA ligand exchanged QDs and QD-DNA probe conjugate with varying ratios of QD:oligonucleotide. The corresponding calculated radiative and non radiative decay values are also shown.
There were no observable spectral shifts between bare MPA QDs and QD-DNA conjugates or between the different ratios of QD-DNA conjugates. The interest was in changes observed after the attachment of oligonucleotide strands rather than variations between different ratios of QD-DNA conjugates. The attachment of oligonucleotide probes involves a change in the surface chemistry available on the QD surface and should play an important role in its spectral properties as well as future biological activity. Passivation from oligonucleotides resulted in an increase in quantum yield (QY) and lifetimes. An approximately 10-fold increase in the quantum yield was observed for QD-DNA conjugates compared to bare MPA QDs. It has been reported that doubling the number of probes resulted in a similar doubling of the quantum yield. There was no significant difference in QY between the different QD:probe conjugates. The variations were attributed to variability in replicate preparations.

Similar trends were observed with lifetimes which increased after the attachment of oligonucleotides to the surface of the QD. Again, there was little variation between the different QD:probe ratios suggesting that the loading capacity of green quantum dots saturated at a ratio of 4 to 5 oligonucleotide strands per QD.

<table>
<thead>
<tr>
<th>QD:DNA conjugate</th>
<th>QY ($10^{-2}$)</th>
<th>Lifetime, $\tau$, (ns)</th>
<th>Radiative decay, $k_r$, ($10^6$ s$^{-1}$)</th>
<th>Non radiative decay, $k_{nr}$, ($10^8$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0:0$^a$</td>
<td>2.044 ± 1.379</td>
<td>9.128 ± 1.087</td>
<td>2.239 ± 1.534</td>
<td>1.073 ± 0.01530</td>
</tr>
<tr>
<td>1:4:0</td>
<td>23.50 ± 13.92</td>
<td>10.33 ± 1.123</td>
<td>22.75 ± 13.70</td>
<td>0.7407 ± 0.1370</td>
</tr>
<tr>
<td>1:5:0</td>
<td>22.86 ± 13.10</td>
<td>12.46 ± 7.914</td>
<td>18.35 ± 15.70</td>
<td>0.6193 ± 0.1570</td>
</tr>
<tr>
<td>1:4:1</td>
<td>24.00 ± 12.82</td>
<td>9.236 ± 1.537</td>
<td>25.98 ± 14.54</td>
<td>0.8229 ± 0.1454</td>
</tr>
</tbody>
</table>

$^a$ bare MPA QDs
There was no observable change in the spectra between different QD:probe ratios. This indicated that the nature of the ligands at the surface of the QD influences the transition from excited state to ground state and not the number of ligands available.\textsuperscript{25}

2.4.6 Förster Distances

Due to minimal variation between different ratios of QD:probe, Förster distances, \( R_o \), were calculated using individual QD:probe lifetimes. The average \( R_o \) was 5.29± 0.02 nm based on a calculated spectral overlap of 7.61± 0.212 x10^{-10} \text{ cm}^6/\text{mol}. A buffer refractive index of 1.34 and an assumed orientation factor of \( \kappa^2 = 2/3 \) were used. This is an accepted value when the orientation of the transition dipole of the acceptor and the exciton of the quantum dot are both expected to be randomized.\textsuperscript{26}

2.4.7 FRET Efficiency

It was expected that 1:5:0 QD-DNA conjugates would exhibit the greatest FRET efficiency since it was the conjugate with the greatest number of hsp23 probe sites available for hybridization. With a greater number of sites available, more acceptors can be positioned around the donor to achieve greater energy transfer efficiency. This model did not take into account the fact that loading capacity may also play a role. An increased amount of oligonucleotides resulted in increased negative charge density. This in turn increased undesired electrostatic forces between negatively charged QD-DNA conjugates and oligonucleotides in bulk solution.

QD:probe conjugates of the ratio 1:4:0 exhibited the largest FRET efficiency, suggesting that 1:4:0 was the optimal QD: oligonucleotide ratio. Based on previous experiments, the observed 65.1± 7.01% was an enhanced FRET efficiency. The enhanced efficiency was an indication that there were still sites available on the surface of the QD for adsorption.

The least efficient system was QD:probe conjugates of the ratio 1:4:1 with a FRET efficiency of 49.8 ± 15.7%. However, this result suggested that the presence of non-complementary probes helped reduce adsorption while maintaining selectivity.
Table 5. FRET efficiencies calculated using Eq. 2.6

<table>
<thead>
<tr>
<th>QD:hsp23:uidA</th>
<th>E (based on τ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4:1</td>
<td>49.8 ± 15.7%</td>
</tr>
<tr>
<td>1:5:0</td>
<td>59.2 ± 24.5%</td>
</tr>
<tr>
<td>1:4:0</td>
<td>65.1 ± 7.01%</td>
</tr>
</tbody>
</table>

2.5 Conclusion

Despite concerns for the instability of thiol capped QDs, bidentate thiol chemistry was used successfully for self assembly of QD-DNA bioconjugates. The QD:probe bioconjugates showed increases in quantum yield and photoluminescence lifetimes compared to QDs without any oligonucleotides. FRET sensitized emission from Cy3 labeled targets was observed upon hybridization. It was proposed that the desired QD:hsp23:probe:non-complementary oligonucleotide uidA ratio was 1:4:1. The presence of an equivalent of an oligonucleotide sequence non-complementary to the target sequence was capable of reducing undesired nonspecific adsorption while maintaining selectivity for the target labeled probe sequence. This was confirmed by evaluating FRET efficiency, dynamic range and limit of detection. It was also reaffirmed through kinetic experiments. With the establishment of a ratio of QD:probe which provided a balance in selectivity and sensitivity, the next step was to evaluate its ability to generate signals in displacement based assays.


Chapter 3
Competitive Displacement of Cy3 Labeled Target Oligonucleotides and Corresponding FRET Intensity Changes

Abstract

Bioprobes based on quantum dots (QDs) as FRET donors were developed using a covalently immobilized 25 base pair (bp) oligonucleotide probe that was hybridized with a partially-complementary Cy3 dye-labeled target. The bioprobes were designed to operate in competitive displacement assays with the intention of a label-free sensing scheme that might operate intracellularly. Displacement was initiated by a 98 bp unlabeled oligonucleotide sequence characteristic of small heat shock protein hsp23. The progress of displacement was monitored by decrease in FRET ratios of the intensity of acceptor (Cy3) to donor (QD). The rate of displacement was enhanced by shortening the pre-hybridized labeled strand, as well as introducing base pair mismatches. QD bioprobes were pre-hybridized with 18 or 16 bp dye labeled strands containing 3 base pair mismatches. Different QD:probe:non-complementary oligonucleotide ratios were evaluated. The FRET efficiencies were observed in the 10-65% range. The implications of higher ionic strength on non-specific adsorption and feasibility of displacement to take place were evaluated. Presence of denaturating agents (formamide and heat) has indicated potential for improved selectivity and sensitivity of the system.

3.1 Introduction

A proposed method for intracellular detection of gene sequences featured the use of CdSe/ZnS nanocrystals known as quantum dots (QDs). QDs were functionalized with dithiol terminated DNA oligonucleotide probes via self-assembly. The resulting QD-DNA bioconjugates used QDs as Fluorescence Resonance Energy Transfer (FRET) donors. Typically, detection of hybridization events using QDs as FRET donors was done by tagging the target sequence with a fluorescent dye whose absorption spectrum had significant overlap with the emission spectrum of the QD. Recent reviews indicate a wide range of bioconjugation techniques as well as signal transduction methods for use of QDs as bioprobes and biosensors.\textsuperscript{1-3}
QDs that offer the capability of in vivo imaging and concurrently the possibility of biomarker recognition have also gained widespread interest. An example of such work involves in vivo cellular studies of transferrin-receptor (TFR) mediated endocytosis utilized QDs. TFR is involved in the delivery and uptake of iron into cells. Each TFR homodimer binds two molecules of iron-bound transferrin (Tfn) at the plasma membrane. QDs were coupled to Tfn (QD-Tfn) and were delivered via TFR-mediated endocytosis. Next, an AlexaFluor was also coupled to Tfn yielding AF-Tfn. One QD-Tfn donor and one AF-Tfn acceptor bind to the TFR dimer for internalization. Energy transfer was visualized by confocal microscopy between QD-Tfn donor and AF-Tfn acceptor conjugates during endocytic trafficking.

The majority of reported transduction schemes for bioassays are not suitable options for in vivo studies due to the need for labeling of the target sequence. One example is work by Bakalova who introduced a QD-siRNA conjugate with the intention of selectively detecting small interfering RNA (siRNA) sequences in mammalian cells using FRET. However, prior to any analysis target mRNA had to be isolated from cells and then subjected to linear amplification in the presence of Cy5-labeled nucleotides.

One approach way to circumvent the need for labeling of the gene fragment of interest would be the use of molecular beacons as bioprobes. Molecular beacons contain partially complementary sequences causing them to fold into a hairpin or stem-loop structure. Conformational changes upon hybridization for sensing applications have been reported with QD-DNA conjugates. Molecular beacons functionalized with amines for attachment were modified with a quencher: 4-(dimethylaminoazo)benzene-4-carboxylic acid (DABCYL) on one end and mercaptoacetic acid (MPA) capped QDs on the other. In the presence of a complementary target, QD photoluminescence (PL) increased as the conformational change increased the QD-quencher distance, resulting in decreased quenching efficiency. The addition of non-complementary targets yielded minimal increase in QD PL. Another application using molecular beacons for label free sensing used an intercalating dye to label hybridized strands, but this again limits applicability to in vivo studies.
Another constraint for selective detection using molecular beacons is related to nucleotide sequence length. Probe sequences tend to be relatively short to facilitate the kinetics of hybridization. Target sequences used in the majority of studies were short segments of the genomic sequences expected \textit{in vivo} (\textit{i.e.}, $<$30 base pairs (bp) compared to genomic sequences expected \textit{in vivo} which are on the 100-1000 bp scale). Long target oligonucleotide strands may have difficulty achieving hybridization with molecular beacons due to steric in achieving a conformational change.

The work in this thesis makes use of linear DNA probes and a displacement strategy is proposed for signal transduction. The proposed bioprobe is a proof of concept system for the eventual intracellular delivery into \textit{Drosophila melanogaster} cells. The construct conceptually begins with a QD-probe DNA conjugate (CdSe/ZnS nanocrystals with emission maxima in the green region), and this is hybridized with a Cy3 labeled target to yield a QD-probe+target system that exhibits FRET sensitized Cy3 emission with high FRET efficiencies (\textit{i.e.} a bioprobe where the labeled target can be displaced by a non-labeled biological target). A schematic illustration of the proposed bioprobe is illustrated in Fig. 3.1.

The probe and pre-hybridized target system must be optimized to promote displacement. Displacement was promoted by ensuring the displacer strand had greater affinity to the probe than the initial target sequence. This was done by designing the targets to have (i) fewer base pair matches than the displacer by means of selection of sequence length, and/or to contain (ii) base pair mismatches to destabilize the initial probe-target dsDNA duplex. Studies of displacement based hybridization between DNA targets and probes has been reported by Baker\textsuperscript{9} and were successful with longer 100 bp oligonucleotides. Baker has shown that complementary target sequences of 15 bp even when embedded in the middle of a 100 bp long strand were capable of achieving displacement, although on a much longer timescale. Lee observed QD PL quenching after complexation with a dye labeled oligonucleotide.\textsuperscript{10} Regeneration of QD PL was achieved by hybridization with unlabeled target DNA. These QD-DNA conjugates were prepared by electrostatic interaction which could affect the affinity, stability and interactions between QD and oligonucleotides.\textsuperscript{10} The loss of FRET sensitized dye emission by cleaving a dye labeled
DNA strand hybridized to QD-DNA conjugates and subsequent regeneration of QD PL has also been reported.\textsuperscript{11} Cleavage of the dye was done by a DNase enzyme. This signal transduction method could have potential in \textit{in vivo} studies by taking advantage of nucleases or other intracellular machinery to disrupt the initial QD-labeled DNA FRET pair.

In the work of this thesis, a 25 bp probe was attached to MPA modified QDs via thiol based chemistry. The probe is diagnostic of hsp23 which is one of four small heat shock proteins well known to be upregulated by \textit{Drosophila Melanogaster} after being subjected to an external stressor. The QD:probe conjugate was pre-hybridized with one of three different Cy3-labeled targets that were selected to provide somewhat weakened dsDNA duplex thermal stabilities. The resulting complexes are identified as QD-probe+target systems. The effect of a 98 base displacer strand, representative of the hsp23 gene, on the QD-probe+target systems was investigated for displacement studies. The use of varying QD: probe ratios was investigated to ensure observations were consistent with hybridization events and not simply adsorption issues known to affect short ligand solubilized QDs. Quantification of the loss of the intensity of FRET over time could be subjective. Therefore, the interest was to track the kinetics and dynamics of the competitive displacement event.
3.2 Reagents and Instrumentation

CdSe/ZnS core/shell semiconductor nanocrystals quantum dots (QDs) in toluene with an emission maximum of 524 nm were from Evident Technologies (Troy, NY, USA). CdSe/ZnS core/shell QDs were also synthesized in-house. These QDs had an emission maximum of 536 nm and were kept in chloroform. 98% 3-mercaptopropionic acid (MPA), 99.% N,N-diisopropylethylamine (DIPEA), tetramethyl ammonium pentahydrate, formamide and Tris(2-carboxyethyl)phosphine (TCEP) were from Sigma Aldrich (Oakville, ON, Canada) and used without further purification. Chloroform and dichloromethane were from Caledon Laboratories (Georgetown, ON, Canada) and used as received.
Water was deionized and purified using a Milli-Q cartridge purification system (Millipore Corp, Mississauga, Ontario). Buffer solutions were prepared using autoclaved deionized water (Tris Borate buffer 50 mM pH 7.6; Borate buffer 50 mM pH 8.4 and 9.5). Buffers with increased ionic strength were prepared by the addition of NaCl to a final concentration of 25 mM.

All oligonucleotides were from Integrated DNA Technologies (Coralville, IA, USA) and were purified by HPLC by the manufacturer. Oligonucleotides were dissolved using autoclaved water and filtered through a 0.2 µm syringe filter prior to use. Nucleotide sequences are listed in Table 1. The hsp23 probe sequence corresponds with the heat shock protein 23 sequence expressed by *Drosophila melanogaster* when subjected to external stress. The hsp23 displacer is a portion of the hsp23 sequence that would be upregulated under conditions of stress. The uidA probe sequence is diagnostic of *Escherichia coli* and was chosen as a non-complementary sequence.

**Table 3.1. Oligonucleotide sequences used in hybridization assays**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probe sequences</strong></td>
<td></td>
</tr>
<tr>
<td>hsp23 probe</td>
<td>5’-/5DTPA/ TTT TAA TTG CTT CAA GAT GAT TCT C -3’</td>
</tr>
<tr>
<td>uidA</td>
<td>5’-/5DTPA/ CTT ACT TCC ATG ATT TCT TTA ACT -3’</td>
</tr>
<tr>
<td><strong>Target sequences</strong></td>
<td></td>
</tr>
<tr>
<td>18bp – FC</td>
<td>5’ - GAG AAT CAT CTT GAA GCA /3Cy3Sp/- 3’</td>
</tr>
<tr>
<td>18bp – 3bpm</td>
<td>5’- GTC TAT CAT CTT GAA GCA /3Cy3Sp/ –3’</td>
</tr>
<tr>
<td>16bp – 3bpm</td>
<td>5’- GTC TAT CAT CTT GAA G /3Cy3Sp/– 3’</td>
</tr>
<tr>
<td><strong>Displacer sequence</strong></td>
<td></td>
</tr>
<tr>
<td>hsp23 displacer</td>
<td>5’- AAC AGC TAA AGC GAA AGT AAC CTA TCA ACA AAA GAA GTT TAT TCT TTG AAG GAG AAT CAT CTT</td>
</tr>
</tbody>
</table>
Ultraviolet-visible (UV-vis) absorption spectra were collected using a Hewlett Packard 8452A Diode Array Spectrophotometer (Hewlett Packard Corporation, Palo Alto, CA, USA). Steady state fluorescence spectra of solutions were collected using a QuantaMaster PTI spectrofluorimeter and analyzed using Felix software (Photon Technology International, Lawrenceville, NJ, USA). Photoluminescence lifetime measurements were done using a time correlated single photon counter (constructed in-house). A GL-302 dye laser (PTI) using DPS (in dioxane) was excited with a GL-3300 nitrogen laser (PTI). Data was analyzed using TMaster software (Photon Technology International, Lawrenceville, NJ, USA).

### 3.3 Experimental

#### 3.3.1 QD-DNA Probe Bioconjugates

QDs were made water soluble by ligand exchange with MPA as described in Chapter 2. The desired ratio between QD and probe for QD:probe bioconjugates were prepared as described in Chapter 2.

#### 3.3.2 Hybridization Assays

QD:probe bioconjugates were prepared as described in Chapter 2 using ratios of the form $x:y:z$ where $x$, $y$ and $z$ represent molar ratios of QD, hsp23 and uidA dithiol modified oligonucleotides respectively. The QD:probe bioconjugates had ratios of 1:4:0, 1:4:1 and 1:5:0. QD concentrations were diluted to between 100 nM to 250 nM for hybridization assays using the desired buffer. A minimum 2:1 molar ratio between target and probe was used. Under these conditions, it was known that there was sufficient target to saturate the QD:probe bioconjugate.
The desired target strand was added to the QD:probe bioconjugate solution. The resulting mixture was placed on a vortex mixer before incubation at room temperature in the dark (to prevent photobleaching of the Cy3 labeled DNA). Control experiments received the corresponding volume of buffer to account for any dilution effects. Fluorescence spectra were typically collected 1, 6, 12 and 24 hours after the addition of target. The extent of hybridization was measured as the ratio of relative intensities between different PL wavelengths; 590-560 nm or 580-560 nm and 558-528 or 558-538 nm for emission maxima 525 nm or 536 nm QD systems, respectively. Excitation was at 405 nm where direct Cy3 excitation was negligible. The observed Cy3 PL was a result of FRET indicating that Cy3 target strands were within close proximity to the QD (ie hybridization or adsorption). Confirmation that hybridization was complete was taken as negligible change in the FRET ratio with time. This was observed to take place between 6-12 hours after the addition of target. All spectra were normalized to the initial QD emission maximum (i.e., QD:probe conjugate in the absence of target).

3.3.3 Competitive Displacement Assays

The hsp23 displacer sequence was added in a 1:1 or 1.5:1 molar ratio of displacer and target. The resulting mixture was placed on a vortex mixer before incubation at room temperature in the dark. Control experiments received the corresponding volume of buffer to account for any dilution effects. Additional control experiments were done where hsp23 displacer was added to solutions not containing any labeled target to observe the effect of the hsp23 displacer. Fluorescence spectra were measured 1, 6, 12, 24 and 48 hours after the addition of hsp23 displacer. The extent of displacement was measured as the ratio of Cy3 PL to QD PL over a fixed range of wavelengths. Excitation was at 405 nm where direct Cy3 excitation is negligible. The observed Cy3 fluorescence can be taken as emerging from the FRET process. All spectra were normalized to the initial QD emission maximum (i.e: QD-probe conjugate in the absence of target as well as displacer).

FRET ratios were used to quantify intensity changes of FRET-sensitized Cy3 fluorescence. This accounted for any instrumental or excitation intensity variations that may have occurred due to the collection of multiple spectra over a period of several days. The sample spectrum was first
background subtracted. A FRET ratio, \( R \), was then used to quantify the amount of FRET-sensitized Cy3 fluorescence using Eq. 3.1 or 3.2.

\[
R = \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{DA} - \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{D} \quad \text{OR} \quad R = \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{DD} - \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{D} (3.1)
\]

\[
R = \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{DD} - \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{D} \quad \text{OR} \quad R = \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{DD} - \left( \frac{\sum_{528}^{558} PL(\lambda)}{\sum_{528}^{558} PL(\lambda)} \right)_{D} (3.2)
\]

The wavelengths chosen were of a spectral region of acceptor PL (Cy3; 560-590 nm or 560-580 nm for QD\textsubscript{525} or QD\textsubscript{536}, respectively) and significant donor PL (QD; 528-558 nm or 538-558 nm for QD\textsubscript{525} or QD\textsubscript{536}, respectively). These wavelength intervals were used to integrate fluorescence intensity signals for all samples. The subscript \( DA \) refers to measurements from hybridization experiments (i.e., QD:probe bioconjugate + Cy3 labeled target, QD donor in the presence of an acceptor). The subscript \( D \) refers to measurements from initial spectra (i.e., QD:probe bioconjugate only, donor only). The subscript \( DD \) refers to measurements from displacement-based experiments (i.e., QD:probe + target + displacer, donor in the presence of an acceptor as well as the displacer). The use of QD\textsubscript{525} or QD\textsubscript{536} resulted in different values for FRET ratios due to different spectral overlaps, but did not affect the trends observed. Ratiometric analysis for FRET based studies featuring QD-DNA systems has been reported previously.\textsuperscript{12}

By observing the fluorescence spectra, changes in FRET ratios could be determined to be the result of decreased FRET sensitized emission of the acceptor Cy3 and/or a regeneration of QD PL. An increase in QD PL would be indicative of regeneration of QD PL after the displacement of the target strand. A decrease in the Cy3 signal would be indicative of a loss of proximity of the Cy3 labeled target strand to the QD suggesting displacement. All spectra were initially background corrected. Next, the spectrum of each sample was normalized to the initial QD
emission maximum (i.e., QD:probe conjugate in the absence of target or displacer). This allowed for equally weighted samples despite differences in initial concentrations and also accounted for any discrepancies that could arise from different excitation intensities or instrumental factors while collecting measurements over a period of several days.

FRET efficiencies, $E$, were calculated using Eq. 3.3 which describes the relationship of $E$ with lifetimes, $\tau$, or quantum yield, $\Phi$ in the presence of an acceptor, (subscripts $DA$) or in the absence of an acceptor (subscripts $D$).

$$E = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{\Phi_{DA}}{\Phi_D}$$

(3.3)

3.3.4 Effects of Denaturants

To investigate the effects of a chemical denaturant, QD:probe conjugates were prepared as described in Chapter 2 in Tris borate buffers (50 mM, pH:7.6) containing 0, 10, 30, 50 or 75% formamide. Hybridization and displacement assays were done as previously described. The 18bp-fully complementary target was used. The target strand was added at least 12 hours prior to measurements to allow for complete hybridization. The displacer strand was added 12 hours prior to measurements.

QD:probe bioconjugates were prepared as described in Chapter 2 to investigate the effects of increased temperature on the bioprobe. An excess of target was added. The solutions were placed on a vortex mixer before incubation for 8 hours to overnight in the dark. The solutions were then heated to 35 or 45°C for 5 minutes using a block heater. The solutions were allowed to cool to room temperature in the dark for at least another 5 minutes prior to collecting fluorescence spectra.

3.3.5 Measuring Lifetimes, Spectral Overlap and Förster Distances

Lifetime measurements, spectral overlap and Förster distance calculations were completed as described in Chapter 2. All measurements containing target sequences were collected a minimum of 24 hours after the addition of the target to ensure complete hybridization.
3.4 Results and Discussion

3.4.1 Choice of Target Strands and Förster Distances

The target sequences that were initially examined were engineered to promote displacement by a sequence that was fully complementary to the probe sequence. The system must be capable of supporting FRET-sensitized Cy3 emission. At the same time, it cannot be so durable that displacement cannot take place. The probe sequence attached to the QD was 25 bp long. The first sequence tested was an 18 bp sequence fully-complementary (18bp-FC) to the probe sequence. The use of a shorter sequence should promote displacement as reduced base pairing interactions thermodynamically reduces the stability of a dsDNA duplex.

Subsequently, 3 base pair mismatches were introduced to the 18 bp sequence (18bp-3bpm) to destabilize the system. It has been reported that mismatches not only reduce the overall binding energy but, can also serve as a toehold region to promote interaction between a probe sequence and displacer sequence.\(^9\) Mismatches served as a nucleation site for a second dsDNA duplex to form. The mismatches were sequential and placed near the end of the strand to reduce promote breathing of the resulting dsDNA duplex. This system had a melt temperature, \(T_m\), comparable to the temperature at which heat shock experiments were generally performed (Table 3.2).

A shorter 16 bp sequence containing the same 3 base pair mismatches was chosen for its lower melting temperature (16bp-3bpm). Although the reduced sequence length resulted in the dye being further away from the QD; the difference was 2 base pairs corresponding to a change of approximately 0.7nm. This change was not expected to have a substantial impact on FRET efficiency considering that the calculated Förster distances were approximately 5 nm. With a \(T_m\) close to the temperature at which \textit{Drosophila} cells were grown, this system was chosen to promote faster displacement kinetics.
Table 3.2. Calculated spectral overlap $J$ and Förster distance ($R_o$) for QD-DNA conjugates with varying Cy3 labeled target strands. $T_m$ were obtained from Integrated DNA Technologies (IDT) Biophysics\textsuperscript{13}

<table>
<thead>
<tr>
<th>Target</th>
<th>$J$ (x10^{-10} cm$^6$/mol)</th>
<th>$R_o$ (nm)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QD$_{525}$</td>
<td>QD$_{536}$</td>
<td>QD$_{525}$</td>
</tr>
<tr>
<td>18bp-FC</td>
<td>7.70 ± 0.212</td>
<td>4.45 ± 0.113</td>
<td>5.3 ± 0.022</td>
</tr>
<tr>
<td>18bp-3bpm</td>
<td>6.88 ± 0.189</td>
<td>5.35 ± 0.139</td>
<td>5.2 ± 0.021</td>
</tr>
<tr>
<td>16bp-3bpm</td>
<td>7.19 ± 0.197</td>
<td>4.22 ± 0.107</td>
<td>5.2 ± 0.021</td>
</tr>
</tbody>
</table>

Förster distances were similar regardless of the target sequence. The sequences were designed to have the dye present on the proximal end. Therefore, the dye was well within the Forster distance and large FRET efficiencies were expected.

3.4.2 Hybridization assays

Different FRET efficiencies were observed for different QD:probe+target systems, corresponding with the stability of the dsDNA duplex. As expected, the presence of mismatches in the target sequence decreased the FRET efficiency and extended the lifetime of the QD. These results were tabulated in Table 3.3. The same trends can be observed in the spectra of QD:probe+target systems in Fig 3.2.
Figure 3.2. Spectra of initial FRET obtained from QD:probe+target systems for varying target sequences. (a) QD₃₃₆ and (b) QD₅₂₅. QD:probe ratios were (i) 1:4:1 and (ii) 1:4:0. QD:probe concentrations were generally between 100-250 nM and Cy3 labeled target was added in excess at concentrations of generally 1 µM. FRET sensitized Cy3 emission was normalized to the QD emission peak.
It was suggested that the reason for greater FRET-sensitized Cy3 emission observed in Fig. 3.2 for QD:probes of the ratio 1:4:0 compared to the ratio of 1:4:1 were a result of adsorption. The use of an oligonucleotide sequence identical in length and also modified with a dithiol but non-complementary to the labeled target sequences decreased the FRET-sensitized Cy3 signal. This suggested a reduction in non-specific adsorption, or a sterically and/or electrostatically hindered QD:probe conjugate, resulting in diminished FRET. The attributes of both cases were discussed in Chapter 2.

**Table 3.3.** Tabulated results of lifetimes, $\tau$, and calculated FRET efficiencies of QD:probe bioconjugates hybridized to Cy3-labeled targets.

<table>
<thead>
<tr>
<th></th>
<th>$QD_{525}$</th>
<th></th>
<th>$QD_{536}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau$ (ns)</td>
<td>$E$ (%)</td>
<td>$\tau$ (ns)</td>
</tr>
<tr>
<td>1:4:0 – no target</td>
<td>10.3 ± 1.12</td>
<td>-</td>
<td>2.82 ± 0.342</td>
</tr>
<tr>
<td>1:4:0 + 18bp FC</td>
<td>3.60 ± 1.12</td>
<td>65.1 ± 21.4</td>
<td>2.50 ± 0.327</td>
</tr>
<tr>
<td>1:4:0 + 18bp 3bpm</td>
<td>5.21 ± 1.93</td>
<td>49.6 ± 19.1</td>
<td>2.82 ± 0.280</td>
</tr>
<tr>
<td>1:4:0 + 16bp 3bpm</td>
<td>6.96 ± 1.30</td>
<td>32.6 ± 7.03</td>
<td>2.15 ± 0.359</td>
</tr>
<tr>
<td>1:4:1 – no target</td>
<td>9.24 ± 1.54</td>
<td>-</td>
<td>3.05 ± 0.489</td>
</tr>
<tr>
<td>1:4:1 + 18bp FC</td>
<td>4.64 ± 1.24</td>
<td>49.8 ± 15.7</td>
<td>2.60 ± 0.296</td>
</tr>
<tr>
<td>1:4:1 + 18bp 3bpm</td>
<td>5.08 ± 0.177</td>
<td>45.1 ± 7.66</td>
<td>3.13 ± 0.482</td>
</tr>
<tr>
<td>1:4:1 + 16bp 3bpm</td>
<td>6.43 ± 0.879</td>
<td>32.4 ± 7.05</td>
<td>2.23 ± 0.257</td>
</tr>
<tr>
<td>1:5:0 – no target</td>
<td>12.4 ± 0.209</td>
<td>-</td>
<td>3.38 ± 0.468</td>
</tr>
<tr>
<td>1:5:0 + 18bp FC</td>
<td>5.09 ± 0.524</td>
<td>59.2 ± 6.18</td>
<td>2.84 ± 1.50</td>
</tr>
<tr>
<td>1:5:0 + 18bp 3bpm</td>
<td>5.33 ± 0.756</td>
<td>57.2 ± 8.17</td>
<td>2.82 ± 0.376</td>
</tr>
</tbody>
</table>
The much shorter lifetimes observed with QD\textsubscript{536} made it more challenging to recognize a significant change in lifetime. Table 3.3 indicated that the more stable a dsDNA duplex, the shorter the lifetime. This correlated with greater FRET efficiencies. A decrease in lifetime corresponded with a shorter timeframe in the excited state. This trend was expected as the more stable a dsDNA duplex, the greater the occurrence of energy transfer resulting in i) a decrease in time spent in the excited state and ii) a smaller population of QDs remaining in the excited state over time.

The spread of FRET efficiencies observed in Table 3.3 between the different target sequences was greatest for QD:probe bioconjugates with the ratio of 1:4:0. The range of FRET efficiencies differed by approximately 30% between a system featuring the 18bp-FC and 16bp-3bpm target sequences. QD:probe bioconjugates with the ratios of 1:4:1 and 1:5:0 exhibited smaller variations in FRET efficiency. The spread between target sequences was approximately 18% and 12% for 1:4:1 and 1:5:0 bioconjugates, respectively. The small range was initially interpreted to be an outcome of steric and/or electrostatic hindrance. However, FRET efficiencies observed in 1:5:0 QD-DNA bioconjugates were greater than efficiencies observed for ratios of 1:4:1 suggesting that it was not a steric or electrostatic issue. If it was a steric issue, it would have been expected that 1:4:1 and 1:5:0 ratios exhibited the same efficiencies, due to lack of space at the surface of the QD. Greater efficiencies observed in 1:5:0 ratios were likely due to the fact that this system had an additional complementary probe available for hybridization. Therefore, sterics were not considered to be an issue. The results confirmed that the presence of a non-complementary probe was capable of reducing non-specific adsorption by passivating the surface of MPA coated QDs. It is likely that the 1:4:0 exhibited enhanced FRET sensitized Cy3 emission arising from adsorption effects. Both 1:4:0 and 1:4:1 QD:probe bioconjugates were used for subsequent analyses.
3.4.3 Displacement Assays

Figure 3.3. Sequence and time-dependent displacement as observed by decreasing FRET ratios (i) 1:4:0 and (ii) 1:4:1 QD-DNA bioconjugates in 50 mM Tris Borate buffers containing (a) no additional NaCl or (b) 25 mM NaCl. It was observed that FRET ratios decreased steadily over time for the 18bp-3bpm target sequence. QD:probe+target systems were denoted by DA for donor in the presence of acceptor. QD:probe+target systems in the presence of the displacer were denoted by DD. FRET ratios DA were calculated after complete hybridization which was taken to be 12 hours. Subsequent time indications refer to the time after the addition of the displacer. Target and displacer sequences were added in excess at 1.5 molar ratio to probe.

Increases in FRET ratios for controls can be observed in Fig 3.3 (no addition of target or displacer nucleotides) were attributed to the effect of dilution resulting in decreased QD PL. Control experiments received equivalent amounts of buffer instead of target or displacer sequences. The effect of dilution decreased the intensity of the QD peak. After the addition of an equivalent volume of target, QD intensities decreased to approximately 97 ± 0.10% of the initial QD intensity. After the addition of an equivalent volume of displacer, QD intensities were
approximately 91 ± 0.08% of the initial QD intensity. Due to a decrease in the denominator of Eq. 3.1 and 3.2 with no corresponding changes in the numerator; an increase in FRET ratio was reported.

It was consistently observed that there was not a significant decrease in FRET for QD:probe bioconjugates that were hybridized with the 18bp-FC target strand (Fig 3.3). This suggested that although this target strand was shorter than the displacer sequence the energetics for displacement were not favourable. This was likely due to the fact that the target sequence was fully complementary and thus the dsDNA duplex was very stable.

The presence of 3 base pair mismatches resulted in steady time-dependent decreases in FRET in the presence of displacer at all ratios (Fig 3.3). This trend was expected based on decreased $T_m$ and stability of dsDNA duplexes containing mismatches. For sequences 14-20 base pairs in length, it is possible to obtain decreases in melt temperature of up to 5°C per mismatch.\textsuperscript{14}

The decrease in FRET ratios also appeared to be faster in conditions with greater ionic strength (Fig 3.3b). This was expected as higher salt conditions result in lateral displacement of base pairs. Furthermore, higher ionic strengths serve to increase entropy and decrease enthalpy. These combined properties help to lower the free energy of the system, further promoting dissociation and increasing the possibility of displacement. Nucleobases slide relative to each other resulting in changes in the diameter of the double helix structure of dsDNA that can range from -0.1 nm to +0.2 nm.\textsuperscript{15} Under conditions with greater ionic strength, the fully complementary target strand also exhibited overall decreases in FRET ratios over time. However, this trend was not as steady or controlled as that observed for the 18bp-3bpm system.

It was expected that the 16bp-3bpm target sequence would exhibit displacement observed as decreased FRET ratios on a faster timescale as the stability of the dsDNA was further
compromised. This was not observed in solutions of lower ionic strength. This was likely due to the fact that the initial hybridization of the 16bp-3bpm target strand did not form a thermodynamically stable dsDNA duplex. A significant amount of breathing was likely to be taking place after the addition of the 16bp-3bpm target strand. After the addition of the displacer strand, increased FRET ratios were observed. These results were attributed to dilution effects as well as an effect of the lower quantum yield of the Cy3 fluorophore compared to QDs. The quantum yield of Cy3-dsDNA conjugates has been reported to be 0.04\(^{16}\) whereas the quantum yield of the QDs used in the work presented in this thesis were approximately 0.22-0.24 as reported in Chapter 2. Due to the large difference in quantum yields, greater distinction would be observed in QDs PL compared to Cy3 emission.

In solutions with higher ionic strength (Fig. 3.3b), after the addition of 16bp-3bpm target distinct FRET sensitized Cy3 emission was observed. This confirmed that the presence of additional ions aided with hybridization efficiency as observed in the kinetics studies of Chapter 2. The FRET ratio was approximately 50% lower than that observed with target strands designed to have greater thermal stability. Upon addition of the displacer, it was observed that the FRET ratio decreased steadily with time. The decrease observed after 6 hours was comparable to the loss observed with 18bp-3bpm target in the same time frame.

QD:probe bioconjugates with the ratio 1:4:1 showed greater decreases in FRET ratios over the same timeframe compared to 1:4:0 QD:probe bioconjugates when hybridized with the 16bp-3bpm target. These results provided further evidence that 1:4:0 QD:probe bioconjugates exhibited enhanced FRET sensitized Cy3 emission as a result of adsorption. It is unlikely that shorter adsorbed oligonucleotides (Cy3-labeled target sequences) would be displaced by a much longer oligonucleotide sequence (the displacer sequence). There was no statistically significant decrease in the rate of decrease of FRET ratios. FRET ratios 6 hours after the addition of the displacer strand (DD) were 52 ± 0.17% of the initial FRET ratio (DA) for 1:4:0 QD:probe bioconjugates. QD:probe bioconjugates of the ratio 1:4:1 exhibited 46 ± 0.053% of the initial FRET ratio in the same timeframe. The net decreases after 72 hours were statistically similar for the two ratios.
It was of interest to observe not only the decrease in FRET ratios which could be achieved by an increase in the QD PL (ie: regeneration of QD PL due to displacement of the target strand) or a decrease in Cy3 emission (ie: loss of proximity of the labeled target strand to the QD which would also be indicative of displacement). As such, spectral analysis was performed to evaluate the role of the numerator, denominator or both.

Tailing in the sub-500 nm region after the addition of the displacer strand was observed in multiple experiments. This could be due to the formation of aggregates. Aggregation has been observed in QD-DNA bioconjugates and is believed to be a result of charge neutralization arising from interactions between the surface of the QD and phosphate backbone of nucleotides.\textsuperscript{10,17} A greater decrease in QD PL was observed in solutions at higher ionic strength. QD PL intensity dropped to approximately 50% of the original intensity after 72 hours compared to approximately 70% of the original intensity for solutions in low ionic strength. This could be attributed to the ability of additional ions to provide some charge shielding and thus allow more oligonucleotides to interact with the surface of the QD. There were no significant differences between 1:4:0 and 1:4:1 QD:probe conjugates.
Figure 3.4 QD:probe conjugates of the ratio (a) 1:4:0 and (b) 1:4:1 pre-hybridized with the 18bp-FC target sequence. Time based decreases in FRET were the result of QD PL regeneration in combination with decreased FRET sensitized Cy3 emission. All spectra were normalized to the initial QD:probe bioconjugate peak (i.e., in the absence of both target and displacer strands). Experiments were done using 100 nM of QD:probe bioconjugates in 50 mM Tris-Borate buffer, pH 7.6 with 25 mM NaCl. The 18bp-FC target strand was added in excess to a final concentration of 1 µM 12 hours prior to obtaining the spectra. The displacer strand was added in excess to a final concentration of 1 µM.

Although decreases in FRET ratios were observed for some cases in systems with the 18bp-FC target strand (Fig. 3.3), regeneration of QD PL was observed for the QD:probe ratio of 1:4:0 but not 1:4:1 (Fig. 3.4). In general, it was observed that both QD PL and FRET sensitized Cy3 emission decreased over time resulting in the decreasing FRET ratios observed. This was consistent with a displacement reaction. There were no significant differences between 1:4:0 and 1:4:1 QD:probe conjugates.
Figure 3.5 Time based decreases in FRET are exhibited in the spectra as the result of QD PL regeneration in addition to decreased FRET sensitized Cy3 emission. QD:probe+target systems were prepared in (a) 50 mM Tris Borate buffer, pH7.6 and (b) 50 mM Tris Borate buffer, pH 7.6 with an additional 25 mM NaCl. QD:probe conjugate ratios were (i) 1:4:0 and (ii) 1:4:1. Experiments were done using 100 nM of QD:probe, target and displacer strands were added in excess to final concentrations of 1 µM.

QD:probe+target systems featuring the 18bp-3bpm target sequence exhibited steady time-dependent decreases in FRET ratios. The decrease was the result of a combination of QD PL regeneration in addition to decrease in FRET sensitized Cy3 emission. These time-dependent trends were consistent over time, with the QD PL increasing to almost the initial QD PL despite the expected dilution effects. Both QD:probe systems exhibited diminished FRET ratios within 6 hours after the addition of the displacer strand. A comparison of QD:probe conjugates of the ratio 1:4:1 versus 1:4:0 showed the latter exhibiting QD PL regeneration within 6 hours after the addition of the displacer strand. QD:probe conjugates of the ratio 1:4:1, on the other hand, did not exhibit QD PL regeneration until 72 hours after the addition of the displacer strand. This may be due to the presence of the non-complementary uidA probe which obstructed the space necessary for the longer and thus larger displacer strand to interact with the complementary hsp23 probe. Greater regeneration was observed for high salt conditions in both QD:probe systems. These results illustrate a drawback of the 1:4:1 QD:probe bioconjugate. While it was
capable of reducing non-specific adsorption, it was also more sterically hindered obstructing the ability of the displacer strand to compete.

It was observed in Fig. 3.3 that FRET ratios increased over time for systems featuring the 16bp-3bp target sequence. The data of Fig. 3.6 indicates that the increase in FRET ratio was not a result of increased FRET sensitized Cy3 emission. Rather, more substantial decreases in QD PL were observed over time compared to minimal changes in Cy3 emission. There was minimal initial FRET displayed by the QD:probe_target system in solutions of low ionic strength. No regeneration of QD PL was observed. These results, combined with a \( T_m \) near the incubation temperature and low FRET efficiency suggested that the QD:probe+16bp-3bp system would not be viable for intracellular studies.

Figure 3.6 QD:probe+16bp-3bp system of the ratio (a) 1:4:0 and (b) 1:4:1. Experiments were done in 50 mM Tris borate buffer, pH 7.6 with no additional salt using 100 nM of QD:probe bioconjugates. Target and displacer strands were added in excess to final concentrations of 1 \( \mu \)M.

In solutions of higher ionic strength however, the QD:probe+16bp-3bp system displayed less FRET sensitized Cy3 emission than the other target sequences that have been studied. However,
it showed faster displacement and equilibration than the other systems: approximately 24 hrs. As such, the QD:probe+16bp3bpm system could be considered a suitable bioprobe system for further consideration in conditions of high ionic strength. There were no statistically significant differences between the two QD:probe ratios.

![Figure 3.7](image.png)

**Figure 3.7.** QD:probe+16bp-3bpm systems of the ratio (a) 1:4:0 and (b) 1:4:1. Experiments were done in 50 mM Tris-Borate buffer, pH 7.6 with an additional 25 mM of NaCl using 100 nM of QD: probe bioconjugates. Target and displacer strands were added in excess to final concentrations of 1 µM.

### 3.4.4 Effects of Formamide

Formamide is a chemical denaturant well known for its ability to disrupt hydrogen bonding interactions. It destabilizes the helical state of dsDNA allowing for lowering of $T_m$ by 2.4-2.9°C per mole of formamide.\(^\text{18}\) For every increase of 1% (v/v) of formamide, the $T_m$ is lowered by approximately 0.72°C.\(^\text{19}\) The extent of destabilization is dependent on the composition (in particular G+C nucleobases), helix conformation and state of hydration.\(^\text{18}\) It has been previously observed that decreased adsorption was observed with increasing amounts of formamide in QD-DNA bioconjugates featuring thiol based ligands for solubility in aqueous solutions.\(^\text{20}\) Although the addition of formamide to a solution of bioprobes destined for entry into cells was not a viable...
option, experiments were performed to investigate the effects of a chemical denaturant on the proposed QD:probe+target system. The results of such a study provided further information concerning the thermodynamic stability of dsDNA duplex. Only the QD:probe+18bp-FC system was subjected to formamide treatment. As the 18bp-FC target sequence has been shown to be the most thermodynamically stable of the 3 target sequences, the effect of a denaturant on the system was of interest.

Figure 3.8. FRET ratios calculated after the addition of 1 µM 18bp-FC target as denoted by DA and after the addition of 1 µM displacer strand as denoted by DD. Oligonucleotide strands were added 12 hours prior to measurements. Percent formamide refers to the percent formamide present in 50 mM Tris Borate buffers at pH 7.6. QD:probe ratios were 1:4:0 and 1:4:1 for (a) and (b), respectively.

Measurements were taken 12 hours after the addition of the displacer sequence. Based on previous experiments, concurrent regeneration of QD PL and decrease in FRET sensitized Cy3 emission have been observed up to 72 hours after the addition of the displacer sequence. This time frame was expected to be representative of the effect of formamide on the system.
Decreasing FRET ratios were observed in Fig. 3.8 as the percentage of formamide increased. In buffer solutions containing 50% (v/v) or more formamide, a substantial drop in DA FRET ratio was observed. The denaturing effect of formamide discourages hybridization. The extent of the decrease in FRET scaled with formamide. The FRET ratio after the addition of displacer (DD) were $98 \pm 0.13$, $97 \pm 3.3 \times 10^{-3}$ and $90 \pm 0.03\%$ of the initial FRET ratio for QD:probe+18bp-FC conjugates of the ratio 1:4:0 in buffers containing 0, 10 and 30% formamide, respectively. For 1:4:1 QD:probe conjugates, DD FRET ratios were $96 \pm 0.08$, $97 \pm 0.01$ and $92 \pm 0.04\%$ of DA FRET ratio in buffers containing 0, 10 and 30% formamide, respectively. The greater decrease observed for 1:4:0 QD-DNA conjugates supported the hypothesis that initial FRET ratios after the addition the labeled target were enhanced due to adsorption.

Spectral analysis indicated that minimal FRET sensitized Cy3 emission was observed in solutions containing more than 50% (v/v) formamide after the addition of the target strand. This corresponds with the low FRET ratios observed in Fig 3.6. At such high concentrations of formamide, the solution was no longer considered a true aqueous solution. These results were beneficial towards the analysis of the design of the QD bioprobe as it indicated the extreme denaturing conditions required to ensure no hybridization between the target strand and the QD-probe conjugate.

The greatest decrease in FRET ratios were observed for solutions containing 30% (v/v) of formamide. Twelve hours after the addition of the displacer, FRET ratios were $90 \pm 0.03$ and $92 \pm 0.03\%$ of the initial FRET ratio for QD:probe ratios of 1:4:0 and 1:4:1, respectively. Based on FRET ratios, QD:probe+target systems prepared in solutions containing a chemical denaturant did not exhibit statistically significant differences in decreased FRET ratios within the same timeframe. FRET ratios calculated 12 hours after the addition of the displacer strand for QD:probe ratios of 1:4:0 and 1:4:1 containing no formamide were $90 \pm 0.05\%$ for the former and $92 \pm 0.07\%$ for the latter of the initial FRET ratio.
3.4.5 Effects of Heating

The melt temperature, $T_m$, of dsDNA reflected the thermodynamic stability of the hybrids. The bioprobes were designed with the intent of eventual intracellular delivery to *Drosophila melanogaster* cells. These cells are grown at 22°C. In response to external stress, most often in the form of heat, specific heat shock proteins are upregulated. Heat shock experiments are typically performed at 35°C. The QD:probe+target systems were subjected to heating to observe any changes in FRET ratios which may arise from heating.

![Graph showing FRET ratios](image)

**Figure 3.9.** Solutions were heated to the desired temperature for 5 minutes and then allowed to cool to room temperature for at least 5 minutes prior to measurements. QD:probe solutions were generally 100 nM and target was added in excess at a final concentration of 1 µM.

Additional temperatures chosen for heating experiments were based on manufacturer reported $T_m$ of Cy3-labeled targets hybridized to the complementary probe sequence as listed in Table 2. No displacer strand was added to these solutions and any changes in FRET ratios were a result of thermal denaturation. Heat shock protocols require slowly cooling cells to the original incubation temperature of 22°C in order to allow for recovery and upregulation of the heat shock proteins. As such, slow cooling was done despite the increased possibility of re-annealing. As expected, the 18bp-FC target sequence did not exhibit significant changes in FRET when heated below the $T_m$. The $T_m$ for the QD:probe+18bp-3bp dsDNA system was approximately 30°C. Steady decreases in FRET ratios were observed in Fig. 3.7 with increasing temperature for this system.
The same trends can be illustrated in the spectral data as shown in Fig 3.8 including the regeneration of QD PL coupled with decreased Cy3 emission.

**Figure 3.10** Spectra of different QD:probe hybridized with target sequences (i) 18bp-FC, (ii) 18bp-3bpm and (iii) 16bp-3bpm in response to heating. Aggregation of QD:probes was observed. QD:probe solutions were generally 100 nM and target was added in excess at a final concentration of 1 µM.

The QD:probe+16b-3bpm dsDNA system had a $T_m$ of approximately 21°C which was below room temperature and the optimal growth temperature of *D. melanogaster* cells. This sequence was initially chosen as it was expected to be less thermodynamically stable and thus would be capable of exhibiting displacement on a faster timescale. However, it has been shown that this system may be too unstable resulting in minimal hybridization between the QD:probe conjugate and target under conditions of low ionic strength. The effects of heating on this system supported this theory. FRET ratios were observed to decrease after heating to 35°C as shown in Fig 3.10. At higher temperatures, the FRET ratios were observed to increase. The increase was likely an effect of decreased QD PL due to heating.
3.5 Conclusion

A proof of concept for a new bioprobe construct has been demonstrated in competitive displacement assays that has made use of QDs as FRET donors in a QD:probe+target system. Cy3-labeled targets were pre-hybridized to QD:probe bioconjugates and exhibited strong FRET sensitized Cy3 emission along with quenching of QD PL. After the addition of an unlabeled displacer strand that was fully complementary to the probe, Cy3 emission was observed to decrease and was correlated with an increase in QD PL. This was considered to be indicative a displacement event.

The Cy3-labeled targets were designed to allow for sufficient thermal stability in the initial QD:probe+target system enabling FRET. However, the labeled targets were also engineered to be shorter and to contain mismatches so as to promote displacement by the fully complementary displacer sequence. An 18bp sequence containing a 3 base pair mismatch indicated a regeneration of QD PL in addition to Cy3 emission reduction. Significant regeneration of QD PL was also observed for a 16bp sequence containing a 3 base pair mismatch in conditions of high ionic strength. Both systems were considered to have the desired properties for prospective bioprobes for potential *in vivo* applications.

The effects of other dsDNA destabilizing agents were investigated. In the presence of formamide, the efficacy of initial hybridization was greatly diminished. Increasing formamide concentrations resulted in greater decreases in FRET ratios. However the decrease in FRET ratios were not significantly different to decreases in FRET ratio observed as a result of the addition of the displacer strand alone. The effect of thermal denaturation of dsDNA duplexes was also investigated. The 18bp-3bpm sequence provided the best analytical performance for determination of the displacing sequence.

Some optimization of the optimal QD:probe ratio was done by comparing 1:4:0 and 1:4:1 QD:probe bioconjugates. It was confirmed that 1:4:1 QD:probe conjugates were not too sterically hindered and that the presence of a non-complementary oligonucleotide sequence identical in length to the probe served to reduce adsorption. It was also observed that the initial FRET sensitized Cy3 emission observed using 1:4:0 QD-DNA bioconjugates may have been enhanced due to non-specific adsorption.


(4) McGrath, N.; Barroso, M. *Journal of Biomedical Optics* **2008**, *13*.


Chapter 4

*In vivo* Analysis of the QD:probe+target Bioprobe

**Abstract**

A QD:probe+target bioprobe featuring QDs decorated with oligonucleotide probes and pre-hybridized with fluorophore labeled oligonucleotide sequences was introduced into *Drosophila melanogaster* cells. Intracellular delivery was achieved through transfection using a cationic lipid based formulation commercially available as Cellfectin® II Reagent. Fluorescence imaging of cells after transfection was done using laser scanning confocal microscopy. QD:probe+target bioconjugates retained their fluorescent properties, and strong fluorescence was observed from QD and FRET sensitized emission from Cy3 dye. It was determined that the QD:probe+target bioconjugates remained sequestered within endosomal packages. With increasing concentrations of bioconjugates, aggregation was observed in addition to increasing migration of the endosomes towards the cell membrane, presumably for exocytosis.

**4.1 Introduction**

The bioprobe that was used in this work was specifically designed to be able to provide transduction of nucleic acid hybridization in an intracellular environment. The intention was to maintain selectivity for oligonucleotide sequences based on RNA association with hsp23 sequences, and to transduce hybridization by means of ratiometric fluorescence intensity determinations using emission from QDs and FRET excitation of Cy3. The study of RNA within cells is useful for understanding the dynamics of synthesis and processing as it relates to gene expression.¹ Intracellular RNA data has largely been derived by *in situ* hybridization of fixed cells, or by application of specialized PCR methods to individual fractions including subcellular components.² Live cell imaging provides the possibility for spatial and temporal resolution.² This includes the ability to observe the dynamics of production and transport of mRNA after stimulating a cell to express a certain gene.
The application of a FRET-based detection method is advantageous due to the distance dependence for FRET. Donor and acceptor chromophores must be in close proximity (nanometer scale) for a significant time for a FRET signal to become significant. The probability of such through-space energy transfer process occurring by coincidence is highly unlikely. When considering application of FRET as a detection method, there are concerns about direct excitation of the acceptor at the excitation wavelength of the donor. This issue was ameliorated by the use of quantum dots (QDs) as FRET donors, offering a broad excitation range and large Stokes’ shift.

A variety of schemes that make use of FRET for nucleic acid detection by hybridization using FRET have been introduced. For example, two complementary strands have been used where each was individually labeled with a donor or acceptor and could hybridize to a longer target sequence. The proximity of the strands upon hybridization resulted in FRET in this sandwich assay scheme. The potential for hybridization of the complementary strands in the absence of the target sequence presented the possibility of false positives. This scheme would also be difficult to apply in vivo due to the inherent instability of single-stranded DNA probes as they would be subjected to degradation by nucleases. Intercalating dyes can also be incorporated into the sample in order to observe fluorescence in the presence of dsDNA. This would ameliorate the issue of single-stranded DNA probes but introduces intercalating dyes in vivo.

Detecting hybridization using a single stranded DNA probe labeled with both the donor and acceptor has also been reported. Hairpin or molecular beacons are oligonucleotide sequences in specific configurations where certain parts of the strand are complementary to each other resulting in a hairpin or stem-loop structure. Labeling opposite ends of the nucleotide with a fluorophore (donor) and quencher (acceptor) resulted in no fluorescence when the nucleotide was in its bound state. Upon hybridization with a fully complementary sequence, the structure was held in a more rigid, linear and open structure typical of dsDNA oligonucleotides. This increased the separation between the fluorophore and quencher resulting in fluorescence from the fluorophore. Thermodynamic evaluation indicated that there were always some nucleotides in varying open configurations, and the consequence is potential for false positives and high
background fluorescence. The intracellular stability has also been investigated where a molecular beacon specific for β-actin mRNA labeled with TAMA (a fluorophore) and a dabycyl quencher was injected into kangaroo rat kidney cells.\textsuperscript{4} Time correlated images showed progressive increases in fluorescence intensity. No increases were observed in the control experiments. This indicated that increased fluorescence could be attributed to hybridization with mRNA for β-actin, and not hydrolysis or intracellular degradation of the molecular beacon.\textsuperscript{4} There have also been reports of non-specific interactions with nuclear proteins and/or nucleic acids as evidenced by identical results when using linear probes that were substituted for the folded structure of a molecular beacon.\textsuperscript{1} The use of a single probe molecule was considered more advantageous as it allowed for quantitative measurements using wavelength ratiometric measurements.\textsuperscript{4}

Earlier examples of fluorescence based studies for nucleic acid detection in cells featured fluorescence in situ hybridization (FISH). FISH was done using fixed cells where chromosomes or specific genes were labeled by means of binding with using fluorescently labeled probe DNA\textsuperscript{4} (after denaturation of the chromosomes). Probes can be labeled with one or more fluorophores and can be complementary to specific genes within the chromosome, multiple chromosomes, the centromeric, or telomeric region of the chromosome.\textsuperscript{4}

A popular method for live cell imaging has featured green fluorescent protein (GFP) imaging where an RNA-binding protein was fused to GFP and expressed along with the target mRNA.\textsuperscript{2} The target mRNA was capable of binding the RNA-binding protein with high specificity and affinity.\textsuperscript{2}

The GFP approach has been combined with QDs: plasmid DNA was conjugated with phospholipid coated QDs.\textsuperscript{6} The QD-DNA conjugates were transfected into Chinese hamster ovary (CHO-K1) cells that were capable of expressing the reporter protein: enhanced GFP (eGFP) with efficiencies comparable to unconjugated plasmid DNA.\textsuperscript{6} A similar report featured QDs conjugated with nuclear localizing signal peptides who also successfully promoted the
expression of eGFP in human embryonic kidney (HEK293) cells. In another study, PEGylated QDs were conjugated with a tumour homing peptide to deliver small interfering RNA (siRNA) designed to achieve knockdown of eGFP. Significant knockdown of eGFP was observed in transfected HeLa cells. This study set the stage for combined therapeutic and imaging possibilities using QD delivery systems by selectively designing the siRNA sequence.

The GFP pathway assumes that GFP-tagged RNA-binding proteins are not overexpressed relative to the amounts of RNA to be detected. The protein and RNA motif should not be naturally expressed by the cell to avoid false positives. Unfortunately, GFP is always fluorescent and it may be difficult to distinguish unbound GFP from GFP bound to the target mRNA. This can be done by expressing low amounts of the GFP fusion protein or by ensuring that the majority of unbound GFP remains in the nucleus and not the cytoplasm where the mRNA is to be imaged.

PUMILIO1 is a RNA binding protein that binds to RNA in a sequence specific manner rather than through secondary structures. It has been used to bind to two adjacent eight nucleotide stretches of an endogenous mRNA encoding a subunit of NADH dehydrogenase allowing for imaging of mitochondria in HeLa cells.

The use of oligonucleotides has advantages over proteins and peptides as it does not require engineering genes to create a tagged mRNA and GFP construct. There is also multiplexing potential as different sequences can be labeled with various dyes, where the limitation would be spectral deconvolution of the fluorescence emission from the dyes. The disadvantages include lower sensitivity of organic fluorophores compared to GFP tags. Furthermore, oligonucleotide probes are subject to intracellular degradation. DNA-RNA hybrids are targeted by RNase H, an enzyme that degrades mRNA in DNA-RNA hybrids. To circumvent this problem, a variety of backbone modified nucleotides have been synthesized. 2’-O-methyl RNA probes have shown resistance to degradation by RNA specific nucleases, as well as faster hybridization kinetics with
target RNA sequences, greater stability (evidenced through higher melting temperatures) and enhanced binding specificity (evidenced by minimal binding to nuclear proteins). It has been consistently reported that probes delivered to the cytoplasm of cells will move to the nucleus within minutes. This was another area of concern and is unfortunately not well understood. It was known that this translocation does not require energy or cytoplasmic factors. Competitive hybridization featured small double stranded oligonucleotides where one strand was labeled with a fluorophore and the other with a quencher. In the double stranded state, the fluorophore exhibited minimal fluorescence. Target mRNA slowly displaced the strand that was labeled with the quencher, allowing for fluorescence imaging. The second-order kinetics of such a process were slow, but could be improved by designing the fluorophore labeled strand to be longer so as to allow for more nucleation sites with the target mRNA. Competitive hybridization probes have been used for mRNA detection in live cells, although not for naturally occurring mRNA. As a model for examination of competitive hybridization, synthetic oligonucleotides were injected into cells. These results illustrated that the intracellular environment supported hybridization.

In vitro studies have shown promising results for the bioprobe design that has been introduced in this thesis. The design is unique in terms of the use of QDs as FRET donors in conjunction with a competitive displacement scheme for nucleic acid detection in vivo. QDs have been introduced to cells largely for labeling and imaging purposes and protein or antibody based interactions. There have also been advancements in therapeutic applications. In this chapter, protocols for the intracellular delivery of QD bioprobes are presented and preliminary results of in vivo sensitizing abilities are discussed.

4.2 Experimental Reagents and Instrumentation

CdSe/ZnS core/shell semiconductor nanocrystals quantum dots (QDs) in toluene were from Evident Technologies (Troy, NY, USA), ($\lambda_{\text{max}}$ 524 nm) and received in toluene. CdSe/ZnS core/shell QDs were also synthesized in-house (green QDs $\lambda_{\text{max}}$ 536 nm and red QDs $\lambda_{\text{max}}$ 605 nm) and were kept in chloroform. 98% 3-mercaptopropionic acid (MPA), 99% N,N-
diisopropylethylamine (DIPEA), tetramethyl ammonium pentahydrate, Tris(2-carboxyethyl)phosphine (TCEP) and Gentamicin were from Sigma Aldrich (Oakville, ON, Canada) and used without further purification. Cellfectin II Reagent was from Invitrogen (Burlington, ON, Canada) and was used as received. Chloroform and dichloromethane were from Caledon Laboratories (Georgetown, ON, Canada) and used as received. CCM3 cell medium was from Fisher Scientific (Ottawa, ON, Canada) and was used as received. Supplemented cell medium was prepared by the addition of 20 µg/mL of gentamicin. Hoechst 33258 was purchased from Molecular probes (Eugene, OR, USA). Stock solutions were prepared as per the manufacturer’s recommendations in autoclaved water to a concentration of 17 µg/mL.

Water was deionized and purified using a Milli-Q cartridge purification system (Millipore Corp, Mississauga, Ontario). Buffer solutions were prepared using autoclaved deionized water (TB buffer 50 mM pH 7.6; Borate buffer 50 mM; pH 8.4 and 9.5). Buffers with increased ionic strength were prepared by the addition of NaCl to a final concentration of 25 mM.

Modified and unmodified oligonucleotides were from Integrated DNA Technologies (Coralville, IA, USA) and were purified by HPLC by the manufacturer. Oligonucleotides were dissolved using autoclaved water and filtered through a 0.2 µm syringe filter prior to use. Nucleotide sequences are listed in Table 1. The hsp23 probe sequence corresponds with one of the heat shock protein sequences (hsp23) expressed by *Drosophila melanogaster* when subjected to external stress. The hsp23 displacer sequence is a portion of the hsp23 sequence that would be upregulated. The uidA oligonucleotide sequence is diagnostic of *Escherichia coli* and was chosen as a non complementary sequence which was identical in length of base pairs. The uidA sequence was in the same orientation and had a dithiol modification on the 5’ end.

**Table 4.1. Oligonucleotide sequences used in hybridization assays**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
</table>
**Probe sequences**

<table>
<thead>
<tr>
<th>Probe sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp23 probe</td>
</tr>
<tr>
<td>uidA oligonucleotide</td>
</tr>
</tbody>
</table>

**Target sequences**

<table>
<thead>
<tr>
<th>16bp – 3bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’- GTC TAT CAT CTT GAA G /3AlexF647N/- 3’</td>
</tr>
</tbody>
</table>

bp = base pair, Base pairs marked in red indicate mismatches.

Imaging was done by laser scanning confocal fluorescence microscopy using Zen 2009 software. Confocal microscopy utilized a LSM510 module from Zeiss. Excitation laser lines included a laser diode at 405 nm, Argon 2 (maximum output 200 mW) with 458, 477, 488 and 514 nm lines (LGK 7812 ML4, Lasos, Germany); HeNe1 (maximum output 5 mW) at 543 nm (LGK 7786P, Lasos, Germany) and HeNe2 (maximum output 15 mW) at 633 nm (LGK 7628-1, Lasos, Germany) as well as a mercury halide lamp (Xcite Series 120Q, Xcite, Mississauga, Canada). Unless otherwise reported, imaging was done using a Plan Apochromat 63x 1.4 Oil Dic Oil immersion lens. The pinhole was limited to one airy unit and gain conditions were kept consistent across all channels. Zen 2009 software was used for acquiring and processing images.

### 4.3 Procedures

#### 4.3.1 Cell Culture

Kc167 cells were kept in culture flasks maintained with serum free CCM3 cell medium supplemented with 20 µg/mL of gentamicin as an antibiotic. Cells were maintained at concentrations of between 1.0x10⁶ – 2.0x10⁷ cells/mL. Cells were incubated at 22°C in plug-sealed, green-capped T-26 flasks from Sarstedt Inc. (Montreal, QC, Canada). Serum free CCM3 cell medium was kept at 4°C and warmed to room temperature before use.

Cell counts were done using a Petroff Hauser Bacteria Counter and a weighted cover slip to ensure no overlap of cells. Cell counts were used to confirm the health and concentration of cells.
Splitting took place every 4 to 5 days. A maximum of 5 splits were performed in each culture flask before seeding to a new culture flask. After seeding to a new flask, cells were generally given a week to reach confluence.

### 4.3.2 Preparation of QD-DNA+target Complex

QD:probe bioconjugates were prepared as described in Chapter 2. Hybridization with the dye-labeled target strand was done at least 12 hours prior to transfection. QD:probe bioconjugate stocks were generally 0.5–1.0 µM. Transfection solutions were prepared by dilution to concentrations generally between 50-500 nM in the desired buffer to achieve a final volume of 95 µL.

### 4.3.3 Cell Preparation for Transfection

A small volume aliquot of cells, generally 0.3 to 0.5 mL, at confluence was added to 1.7 mL centrifuge tubes. The number of cells was generally in the range of 5.0 - 6.0x10⁶ cells. The cells were centrifuged at 1000 rpm for 4 minutes to achieve a loose pellet. The supernatant was removed and the cells were resuspended in antibiotic free medium. This washing was repeated 3 times before the cells were diluted to a total volume of 0.9 mL in antibiotic free medium in a 15 mL screw cap sterile Eppendorf tube. It was expected that some portion of the total number of cells was lost during each washing step. The cells were given a minimum of 48 hrs to acclimatize to the new environment and antibiotic free medium prior to transfection. Cells were kept at 22°C.

Cell counts were done prior to the addition of transfection solutions to ensure health and concentration, in addition to ensuring absence of contamination. A minimum of 1.5x10⁶ cells was confirmed prior to transfection. Ideal conditions were determined to be approximately in the range of 3.0–5.0x10⁶ cells. Cells prepared for transfection were kept in antibiotic free medium as the transfection of antibiotics can result in cell death.

### 4.3.4 Transfection

A total of 5 µL of a 1 mg/mL solution of Cellfectin ® II Reagent was added to the transfection solutions. Solutions were briefly placed on a vortex mixer and were allowed to incubate at room temperature for a minimum of 15 minutes. It was determined experimentally that 15-25 minutes was sufficient incubation time for the formation of QD decorated liposomes and successful
transfection. The transfection solutions were then added to the cell solutions and mixed by slowly cycling through pipetting. The transfected cell solutions were allowed to incubate at 22°C for 5-8 hours prior to heat shock and/or imaging.

4.3.5 Imaging

A small aliquot of the transfected cell solution was placed into a 1.7 mL centrifuge tube. The volume depended on the concentration of cells. If concentrations were less than 2.0x10^6 cells, 500 µL was removed. For cell concentrations in the optimal range, 100-250 µL was removed. The cells were centrifuged at 2000 rpm for 4 minutes to achieve a pellet. The cells were then washed once using the desired buffer.

Hoechst 33258 stain (stock solutions of 17 µg/mL were prepared according to manufacturer’s directions using autoclaved water) was added to cells that were re-suspended in aqueous buffer for a final concentration of 0.5-1.0 µg/mL. The cells were then incubated for 15-20 minutes at room temperature in the dark prior to washing by centrifugation at 2000 rpm for 4 minutes.

The cells were re-suspended in a minimal amount of buffer (generally 20-30 µL) to yield high cell densities for imaging. A volume of 10 µL of cell suspension was spotted onto glass microscope slides and a glass cover slip was placed on top. The edges of the cover slip were sealed using clear nail polish.

4.3.6 Heat shock

The desired amount of transfected cell solution was placed into a 1.7mL centrifuge tube. Generally 500 µL was transferred. The tubes were placed in a water bath heated to 35-37°C for 30 minutes. The tubes were then removed from the water bath and cooled to room temperature for at least 90 minutes to allow for recovery and continued expression of the heat shock genes. The cells were then prepared for imaging as described previously.
4.4 Results and Discussion

4.4.1 Shift to Red QDs

Autofluorescence issues were of concern for Kc167 cells, which were displayed autofluorescence in the 500-550 nm region when excited at 405 nm. This wavelength range coincided with the wavelengths of interest for green QD emission. Excitation at 458 nm resulted in substantial decrease in autofluorescence but was accompanied by significant losses in QD photoluminescence (PL), as well as by some direct excitation of Cy3. A revised approach made use of the QD-DNA+target system based on red emitting QDs. Red QDs were synthesized in-house and had an emission maximum at 605 nm. The chosen corresponding FRET dye for red QDs was AlexaFluor647. While use of Cy5 was another option, it was determined experimentally that Cy5 had lower FRET efficiencies.

Table 4.2. Imaging specifications.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Emission filter</th>
<th>Excitation source**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst stain</td>
<td>BP475-525nm</td>
<td>LD 405nm</td>
</tr>
<tr>
<td>QD*</td>
<td>LP585nm</td>
<td>LD 405nm</td>
</tr>
<tr>
<td>QD-543</td>
<td>BP585-615nm</td>
<td>HeNe1 543nm</td>
</tr>
<tr>
<td>FRET</td>
<td>LP650</td>
<td>LD 405nm</td>
</tr>
<tr>
<td>Direct excitation of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AlexaFluor647</td>
<td>LP650</td>
<td>HeNe2 633nm</td>
</tr>
</tbody>
</table>

*unless otherwise denoted, all images in the QD channel were obtained using this configuration.

** Laser power was kept between 3.0-5.0% for all images. Within each experiment, the laser power used was identical.
4.4.2 Hoechst 33258

Hoechst dyes are cell permeable nucleic acid stains, making them practical for co-localization studies. The fluorescence of Hoechst dyes is sensitive to DNA conformation and the state of chromatin in cells.\(^{23}\) The dye works by binding to the minor groove in dsDNA. It is particularly selective for A-T base pairs, exhibiting fluorescence approximately 2 times greater in A-T rich dsDNA than in G-C rich dsDNA; but will bind to all nucleic acids.\(^{24}\) Hoechst dyes require a \([\text{dA-dT}]-[\text{dG-dC}]\) sequence to enhance fluorescence.\(^{24}\) The positive charges associated with the structure of Hoechst 33528 undoubtedly also play a role in binding with negatively charged DNA. Dye not bound to dsDNA will exhibit fluorescence in the 510-540nm range. Optimal binding occurs at pH 7.4 and can be achieved in any medium not containing phosphates. Hoechst 33258 was the more readily water soluble of the commercially available Hoechst dyes. It has also been used extensively for staining live cells. Although Hoechst 33258 exhibited greatest spectral absorption in the UV range, the absorption spectra does extend to \(\sim 425\) nm, making it possible to excite using the 405 nm line of the laser diode associated with the confocal microscope.

![Molecular structure of Hoechst dye 33528.](image)

**Figure 4.1.** Molecular structure of Hoechst dye 33528. Note the positive charges associated with the structure that play a role in binding with negatively charged dsDNA.

4.4.3 Use of Transfection Reagents: Cellfectin® II

Cellfectin® II Reagent is a cationic lipid based formulation. The reagent was designed to increase cell permeability promoting endocytosis. It has been used extensively for the introduction of DNA into insect cells as well as adherent and suspended mammalian cells.
To assess whether or not *Drosophila melanogaster* Kc cells would passively uptake QDs, transfected cells differing only in the presence or absence of Cellfectin® II reagent were visualized after approximately 5 hours incubation. Transfected solutions contained QD₆₅₀-probe conjugates with QD:probe ratio of 1:6. QD:probe conjugates were negatively charged due to the presence of oligonucleotides decorating the surface of the QD. Based on electrostatic interaction, they readily form QD-cellfectin complexes. Without the presence of a cationic liposomal package for delivery, it was expected that cells would not exhibit any uptake of QDs. Electrostatic repulsion between the QD-probe conjugates and negative receptors and lipids present on the cell membrane would hinder uptake. As expected, no QDs were observed intracellularly when no Cellfectin® II reagent was added to the transfection solution (Fig 4.2c). This confirmed the need for facilitated delivery for QD:probe conjugates.

Hoechst stain was used as it was known to stain nucleic acids reliably and effectively. The staining procedure provided an indication that the QDs were delivered intracellularly and not just onto the cell membrane surface. The Hoechst stain also aided in determining the location of QDs within the cell. Z-scans were also performed to confirm the presence of QDs within the cell (Fig. 4.2).

Figure 4.2. LSCM images of (a) control (i.e.; transfection solution consisted only of buffer and Cellfectin® II Reagent, (b) with cellfectin and (c) no cellfectin. The channels were (i) white light image, (ii) red QD channel (iii)
Hoechst stain channel and (iv) co-localized image. The images presented were all acquired at the same z-axis position.

4.4.4 Transfecting Varying Concentrations of QDs

To determine the extent of uptake by the cells, varying concentrations of bare QD$_{605}$ (MPA coated, no probe) were incubated with Cellfectin® II to yield the transfection solution. The transfection solution was then incubated with cells for approximately 5 hours. The cells were stained with Hoechst dye to confirm the location of the dsDNA. It was expected that the amount of QDs observed intracellularly would scale with the amount of QD introduced.

It was observed that as QD concentrations in the transfection solution increased, the amount of QDs visualized within the cell also increased (Fig 4.3). However, it was difficult to objectively quantify the presence of QDs. Although efforts were made to keep laser power and gain constant during imaging, there were subtle variances in focus for each slide. This was a result of slide preparation and may have played a role in the intensity of fluorescence detected.

Comparisons were made in terms of the localization of QD PL, the number of cells exhibiting QD PL and the presence of aggregation. The small, confined areas of PL observed in the QD channel suggest that QDs did not form large aggregates until at higher concentrations. Aggregates were observed in cells transfected with 500 nM of QDs (Fig 4.3e). At concentrations below 500 nM, QD PL appeared as distinct spots distributed within the cell. The images suggested that the QDs remain sequestered in their endosomal vesicles after transfection. This was not surprising as there was no additional functionality that would encourage their endosomal escape.

The localization of QDs also changed as a function of concentration. Concentrations above 100 nM resulted in the presence of QDs more towards the edges of the cell. This indicated that not only did QDs remain sequestered within their endosomal vesicles but they had also been recognized as foreign objects and were sent towards the edges of the cells for exocytosis (removal from the cell). Although not shown in Fig. 4.3, it was observed that as concentrations increased, a greater percentage of cells were observed to exhibit QD PL. The results suggested that there was a threshold to the amount of QDs that may be introduced into a cell.
Figure 4.3. LCSM images of (a) control (i.e., transfection solution consisted only of buffer and Cellfectin® II reagent), (b) 50 nM QD, (c) 100 nM QD, (d) 250 nM QD and (e) 500 nM QD. Channels visualized were (i) white light image, (ii) red QD channel, (iii) Hoechst stain channel and (iv) co-localized image.


4.4.5 Intracellular Stability of the QD-probe+target System

To assess the possibility of signal transduction inside cells, a QD:probe+target system featuring 1:4:0 QD:probe was pre-hybridized with a 16bp-3bpm AlexaFluor647 (A647) labeled target prior to transfection. The ratio was in the form of QD:hsp23 probe:uidA oligonucleotide. The merits of different QD:probe ratios and different target sequences were previously discussed in Chapters 2 and 3. Parallel control transfections featured 1:4:0 QD-probe bioconjugate only, 16bp3bpm AlexaFluor647 target only and buffer only. Transfection solutions were prepared to a
final concentration of QD bioprobe of 100 nM. The buffer transfection solution was prepared using the same volume of the desired buffer.

Figure 4.4. LCSM images of (a) control (i.e.; transfection solution consisted only of buffer and Cellfectin® II Reagent), (b) QD-probe conjugate system, (c) 16bp-3bp target labeled with A647, (d) QD-probe+target system. In channels (i)QD-405 nm excitation, (ii) QD-543 nm excitation, (iii) FRET, (iv) direct excitation of AlexaFluor647, (v) co-localization of white light image and QD-405 nm excitation, (vi) co-localization of white light image and QD-543 nm excitation, (vii) co-localization of white light image and FRET and (viii) co-localization of white light
image and direct excitation of AlexaFluor647. There were subtle differences in the intensity of the white light images; this was a result of differing filter sets and laser output between the channels. Transfection solutions were all prepared to have QD:bioprobe concentrations of 100 nM.

It was observed that QD:probe bioconjugates remained stable intracellularly. QD PL was observed when excited with LD 405 nm. FRET sensitized emission of the AlexaFluor647 dye was also observed with LD 405 nm excitation. The results illustrated in Fig. 4.4d indicated that the fluorescence properties of the QD:probe+target system were retained in the intracellular environment.

Due to the technical design of the confocal microscope, the only appropriate emission filter for visualizing QD PL when exciting with 405 nm was a long pass 585 nm filter, and this channel could accommodate FRET emission. As a result, QD channel images were also obtained using a HeNe1 543 nm excitation source coupled with a band pass 585-615 nm emission filter. Unfortunately, exciting QDs at 543 nm did not result in high QD PL. These images served as controls to ensure that no FRET was observed in cells that did not contain the labeled target strand.

The uptake of the labeled target strand was not easily visualized by confocal imaging. Some fluorescence was observed with direct excitation of A647. Rationalization for this observation included insufficient concentrations for visualization, the target strand escaped from the endosomes and was damaged by nucleases present in the cell or the target strand escaped from endosomes and was not damaged by nucleases but other factors within the cytoplasm have quenched the emission. The second postulate seems the most plausible as DNA is known to be prone to degradation by nucleases.\(^1\)

The most significant result was the fact that the QD:probe+target system was intracellularly stable as evidenced by the strong FRET signals observed in Fig 4.4d. Furthermore, the fluorescence emission remained localized within the cell and not just near the cell membrane. Unfortunately, the QD:probe+target system appeared to remain sequestered in endosomal vesicles. Diffuse signals localized throughout the entire cell would be expected for QD:probe+target bioprobes that that were free within the cytoplasm.
4.4.6 Preliminary Heat Shock Experiments

The following sets of images were obtained from the batch of cells imaged in Figure 4.4, but where an aliquot of the cells had been subjected to heat shock. A decrease in FRET signal or increase in QD signal could suggest displacement of the labeled target strand by the mRNA hsp23 sequence expressed as a result of heat shock.

Figure 4.5. LCSM images of cells after heat shock (a) control (i.e.; transfection solution consisted only of buffer and Cellfectin® II Reagent), (b) QD-probe conjugate system, (c) 16bp-3bp target labeled with A647, (d) QD-
probe+target system. In channels (i) QD-405 nm excitation, (ii) QD-543 nm excitation, (iii) FRET, (iv) direct excitation of AlexaFluor647, (v) co-localization of white light image and QD-405 nm excitation, (vi) co-localization of white light image and QD-543 nm excitation, (vii) co-localization of white light image and FRET and (viii) co-localization of white light image and direct excitation of A647. There were subtle differences in the intensity of the white light images, this was a result of differing filter sets and laser output between the channels. All transfection solutions were prepared to a final concentration of QD bioprobe of 100 nM. The cells imaged here were an aliquot of cells imaged in Fig. 4.4 which had been subjected to 30 minutes of heat shock at 37°C. All scale bars were 10 µm.

After heat shock, QD-probe bioconjugates were observed to retain their fluorescent properties within the cell (Fig 4.5b). For QD-probe+target systems (Fig 4.5d), neither a consistent decrease in FRET or regeneration of QD PL was observed. Fluorescence was observed in QD, FRET and direct excitation of A647 channels. Fewer cells exhibited fluorescence compared to what was observed prior to heat shock. However the cause of this reduction of fluorescence intensity cannot be attributed to a change in QD PL or FRET. Assessing the decrease in FRET or regeneration of QD PL based on the intensity of LSCM images was subjective and not an accurate or reliable method of obtaining quantitative data.

It takes time not only for the cells to recover from heat stress but also to express the heat shock protein. It has been reported that expression of hsp begins shortly after the stressor and continues with maximum levels occurring between 2-8 hrs after heat shock. The time at which hsp23 mRNA was most abundant in the cell was expected to be on a shorter timeframe than that of protein expression. As such, it was still necessary to determine the optimum timeframe between the onset of heat shock and detection in order to fully assess the designed bioprobe. This was just one of the many parameters which remain to be optimized. These will be further discussed in Chapter 5.
4.5 Conclusion

The preliminary studies of the bioprobe *in vivo* yielded just as many questions as answers. Successful intracellular delivery of the QD-probe conjugates as well as the QD-probe+target system was achieved. In addition, the QD-probe conjugate and QD-probe+target system retained strong QD PL signals as well as FRET sensitized emission of AlexaFluor647 over extended periods of time. These results indicated the intracellular stability of the QD-probe+target system. However, the location of the QD bioconjugates appeared to vary. Imaging results suggested that the bioprobe remained sequestered in endosomal vesicles as fluorescence was confined to small areas and not diffuse throughout the cell. Co-localization studies with a nucleic acid stain, Hoechst 33258, confirmed that the probe or conjugate systems were not sequestered in the nucleus. There was evidence to suggest that at concentrations greater than 250 nM, there was a possibility of formation of aggregates. Furthermore, as the concentration increased, QD PL was localized near the cellular membrane. This suggested that materials were being transported for the process of exocytosis. Transfected cells subjected to heat shock experiments did not exhibit substantial decreases in FRET or regeneration of QD PL. As a result, it was difficult to quantitatively assess the efficacy of the designed bioprobe.


(15) Smith, A. M.; Duan, H. W.; Mohs, A. M.; Nie, S. M. *Advanced Drug Delivery Reviews* **2008**, *60*, 1226.


(18) McGrath, N.; Barroso, M. *Journal of Biomedical Optics* **2008**, *13*.


5. Conclusions and Future Work

5.1 Conclusions

*In vivo* studies of nucleic acids are of interest to understand the workings of RNA synthesis and processing as it relates to gene expression. Cells can be subjected to a variety of stressors in order to induce the expression of certain genes. The possibility of probing the dynamics of gene expression has far reaching consequences in further understanding the inner workings of organisms on the molecular level. The work of this thesis introduces a displacement assay for nucleic acid detection featuring quantum dots (QDs) as fluorescence resonance energy transfer (FRET) donors. A preliminary investigation of the potential for application of this bioprobe for intracellular studies has been completed.

QDs are used as FRET donors and are covalently conjugated to oligonucleotide probes to yield QD-probe bioconjugate. Optimization of the QD:probe ratio was desired where the bioconjugate was selective for the target and would not generate false signals from non-specific adsorption. The introduction of a non-complementary oligonucleotide probe in bulk solution assisted in saturating the QD surface with oligonucleotide after the probe oligonucleotide was covalently hybridized. Such adsorbed non-complementary oligonucleotide was capable of reducing non-specific adsorption without hindering the selectivity of the QD-probe bioconjugate. The optimal QD:complementary probe: non-complementary probe ratio was determined to be of the form 1:4:1.

The goal of *in vivo* determinations of nucleic acid sequences necessitated development of a bioprobe where signal transduction was based on the displacement of the labeled pre-hybridized target with an unlabeled target strand. The QD-probe bioconjugate was therefore hybridized with a target oligonucleotide labeled with a dye to complete a FRET pair yielding a QD-probe+target system (i.e. the bioprobe). It was determined that saturation of hybridization with target strand to construct the bioprobe was obtained at a ratio of 1:2 probe:target ratio.
To learn how to tune the degree of displacement of the label, a variety of QD-probe+target systems were developed where mismatches and sequences of different lengths were introduced in order to vary the stability of hybrids. *In vitro* studies included investigation of a longer, unlabeled oligonucleotide sequence that contained the complementary target sequence. It was observed that the kinetics of displacement was related to the stability of the hybrid associated with the QD-probe+target system.

Displacement was confirmed by a decrease in FRET sensitized dye emission intensity and a corresponding increase in QD fluorescence intensity. The use of a short target strand that contained mismatches in the construction of the QD-probe+target system resulted in minimal initial hybridization and therefore a lack of initial FRET signal. An improved selection of probe-target strands suitable for displacement assays featured a somewhat shortened target sequence containing mismatches for instability. The displacement was shown to be sensitive to denaturants such as formamide, and to heating as expected in terms of competitive displacement reactions.

The demonstration of signal development in displacement assays *in vitro* suggested that an initial investigation of the bioprobe *in vivo* was warranted. The QD-probe+target system was introduced to cells for *in vivo* studies. Successful intracellular delivery was achieved through transfection via Cellfectin® II reagent, which is known to provide cellular penetration by endocytosis. QD-probe+target systems were observed to remain stable within cells and retained their desired fluorescence properties. Preliminary experiments subjected transfected cells to heat shock to induce the expression of the desired heat shock gene. Unfortunately, the bioprobes remained sequestered in endosomal vesicles and were not free to experience the cytoplasm where the mRNA target associated with heat shock expression was located.
5.2 Future Work

Assessment of the functionality of the bioprobe will require further studies of its intracellular response. Although preliminary co-localization studies using a nucleic acid stain were performed, it was by no means definitive as to the location of QDs after transfection. It is also necessary to confirm whether or not QDs and the corresponding bioconjugates remain sequestered in endosomal vesicles. The addition of an endosomal escape mechanism such as cell penetrating peptides\(^1,2\) should be studied for its efficacy and compatibility with the existing system. An alternative delivery method to avoid the use of Cellfectin \(^\circledR\) II reagent and formation of vesicles is delivery via electroporation.

Once it can be determined that the designed bioprobe is available to function within the cytoplasm, further optimization of performance is necessary. The onset of gene expression after induction by stress is relatively fast.\(^3-6\) As such, the timeframe between stress induction and detection should be optimized. To assess this timeframe, kinetic studies should be performed to study the interactions of the bioprobe in the cytosol environment as a function of time.

Current assessment of the performance of the bioprobe was largely based on subjective comparisons in terms of changes of intensity of fluorescence that was observed. To better quantify data, it is desirable to introduce an internal standard that is also capable of exhibiting fluorescence. A ratiometric methodology will help overcome some of the spectroscopic limitations that are associated with use of fluorescence as a quantitative method in living cells. Finally, as the verdict on the toxicity of nanoparticles remains heavily dependent on surface functionalization in addition to the cell line studied, it is necessary to assess the toxicity the bioprobe.
Should the bioprobe prove to be functional for intracellular studies then there is potential for multiplexing. QDs of different colours can be conjugated to different probe sequences concurrently targeting different genes within a cell. The limitations of such a design are largely dependent on the spectral overlap of dye emissions and the limitations in the hardware available for imaging. A natural extension would be to attach proteins capable of targeting specific organelles for dual imaging and detection possibilities.


