Designing a Matrix Metalloproteinase-7-Activated Quantum Dot Nanobeacon for Cancer Detection Imaging

by

Hsiang-Hua Andy Hung

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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2008

Abstract

Quantum Dot (QD) nanobeacons distinguish themselves from molecular beacons with the promise of non-linear activation, tunability, and multi-functionality. These unique features make them highly attractive for cancer detection imaging with opportunities for increased signal-to-background ratio and tunable sensitivity. In this thesis, a nanobeacon was designed to target matrix metalloproteinase-7 (MMP-7), known to be over-expressed by a wide array of tumours. The nanobeacon is normally dark until specifically activated by MMP-7. The overall design strategy links single QDs to multiple energy acceptors by GPLGLARK peptides that can be cleaved specifically by MMP-7. However, design details such as the choice of energy acceptor and conjugation method was found to drastically alter the function of the nanobeacon. Studies of nanobeacons synthesized with Black Hole Quencher-1 or Rhodamine Red by either covalent conjugation or electrostatic self-assembly revealed that peptide conformation and bonding flexibility are both important considerations in nanobeacon design due to QD sterics.
Acknowledgments

I would like to express my most sincere gratitude to my supervisors Professor Warren Chan and Professor Gang Zheng for giving me this invaluable opportunity to pursue research in an area of my passion. This thesis would not have been the same without their guidance, patience, and encouragements. They have taught me how to be a better scientist, and given me many moments of inspiration.

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# Table of Contents

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

Table of Contents .............................................................................................................................. v

List of Tables ...................................................................................................................................... vii

List of Symbols and Abbreviations .................................................................................................... viii

List of Figures ..................................................................................................................................... ix

List of Appendices ............................................................................................................................ xii

**Chapter 1 Introduction** .................................................................................................................. 1

**Chapter 2 Background** .................................................................................................................. 6

- 2.1 Quantum Dots ........................................................................................................................... 6
- 2.2 Poly(acrylic acid) for QD Coating ............................................................................................. 11
- 2.3 Matrix Metalloproteinase-7 as a Cancer Target ........................................................................ 13
- 2.4 Potential False Positives ......................................................................................................... 16
- 2.5 Förster Resonance Energy Transfer ......................................................................................... 19
- 2.6 Recent Advances in Optical Protease Probes ......................................................................... 21

**Chapter 3 Materials and Methods** .............................................................................................. 26

- 3.1 Materials ................................................................................................................................... 26
- 3.2 Nanobeacon Synthesis Protocol ............................................................................................... 30
- 3.3 HPLC Characterization Methods ............................................................................................. 31

**Chapter 4 Electrostatic Self-Assembly of QD-Peptide Bioconjugates** ........................................ 33

- 4.1 Introduction ............................................................................................................................... 33
- 4.2 Characterization of Binding Constant ....................................................................................... 37
- 4.3 Binding Stability Assessment .................................................................................................... 45
- 4.4 Conclusion .................................................................................................................................. 51

**Chapter 5 QD-BHQ1 Nanobeacon** ............................................................................................. 52

- 5.1 Synthesis Characterization ........................................................................................................ 52
- 5.2 The Effect of Peptide Conformation on Cleavage Efficiency .................................................. 55
- 5.3 Förster Theoretical Analysis ...................................................................................................... 67
- 5.4 Michaelis-Menten Analysis ....................................................................................................... 70
- 5.5 Discussion on Nanobeacon Dissociation .................................................................................. 80
5.6 Conclusion ........................................................................................................................ 83

Chapter 6 QD-Rhodamine Nanobeacon ............................................................................. 84

6.1 Synthesis Characterizations .......................................................................................... 85
6.2 Effect of Conjugation Method on Cleavage Efficiency ................................................ 89
6.3 Discussion ...................................................................................................................... 98
6.4 Conclusion ...................................................................................................................... 102

Chapter 7 Conclusion ....................................................................................................... 103

References .......................................................................................................................... 106
List of Tables

Table 2.1  A comparison between QD and Rhodamine Red-X................................................................. 8
Table 4.1  Example calculation for total and incremental peptide binding percentage.................. 44
Table 5.1  Förster analysis of donor-acceptor separation distances and FRET efficiencies........ 68
Table 6.1  Enzymatic cleavage of different nanobeacons................................................................. 98
# List of Symbols and Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BHQ1</td>
<td>Black Hole Quencher-1</td>
<td></td>
</tr>
<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
<td></td>
</tr>
<tr>
<td>DHLA</td>
<td>Dihydrolipoic acid</td>
<td></td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
<td></td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl Formaldehyde</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
<td></td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
<td></td>
</tr>
<tr>
<td>FRET</td>
<td>Förster Resonance Energy Transfer</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
<td></td>
</tr>
<tr>
<td>$K_b$</td>
<td>Binding constant</td>
<td></td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
<td></td>
</tr>
<tr>
<td>$k_m$</td>
<td>Michaelis-Menten constant</td>
<td></td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
<td></td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
<td></td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid)</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
<td></td>
</tr>
<tr>
<td>QD</td>
<td>Quantum Dot</td>
<td></td>
</tr>
<tr>
<td>Rho</td>
<td>Rhodamine Red-X</td>
<td></td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
<td></td>
</tr>
<tr>
<td>TOPO</td>
<td>Trioctylphosphine</td>
<td></td>
</tr>
</tbody>
</table>
List of Figures

Fig. 1.1  Theoretical plot of energy transfer efficiency as a function of quenchers per QD........ 3
Fig. 1.2  Hypothesized nanobeacon fluorescence activation curves........................................ 4
Fig. 1.3  Nanobeacon concept.................................................................................................. 5
Fig. 3.1  Absorption spectrum and structure of Black Hole Quencher-1 ................................. 26
Fig. 3.2  Absorption and emission spectra and structure of Rhodamine Red-X..................... 27
Fig. 3.3  Structure of Tween 80. ............................................................................................ 28
Fig. 4.1  Gel electrophoresis characterization of electrostatic binding.................................... 37
Fig. 4.2  Measuring QD-Peptide\(^1\) binding constant by monitoring FRET efficiency .......... 38
Fig. 4.3  Percentage peptide binding as a function of QD concentration ............................... 40
Fig. 4.4  Binding curves and fitted dissociation constants for different QD:peptide ratios....... 43
Fig. 4.5  Effect of detergent on QD-peptide self-assembly by fluorescence ............................ 45
Fig. 4.6  Effect of detergent on QD-peptide self-assembly by gel electrophoresis .................. 46
Fig. 4.7  Stability of self-assembled QD-peptide\(^2\) in different buffers.................................. 47
Fig. 4.8  Characterizing mechanisms of QD-peptide self-assembly..................................... 48
Fig. 4.9  Salt stability of self-assembled QD-Peptide\(^2\) ............................................................. 50
Fig. 5.1  Chromatogram of BHQ1-GPLGLARK..................................................................... 52
Fig. 5.2  QD-BHQ1 nanobeacon characterized by quenching and absorbance....................... 53
Fig. 5.3  QD-BHQ1 nanobeacon characterized by HPLC....................................................... 54
Fig. 5.4  Spectral overlap of QD and BHQ1 ........................................................................... 54
Fig. 5.5  Effect of Tween 80 on nanobeacon quenching and cleavage.................................. 57
Fig. 5.6  Tween is necessary for peptide cleavage................................................................. 58
Fig. 5.7  Injecting Proteinase K and Tween in different orders does not change final QD
     fluorescence.................................................................................................................. 58
Fig. 5.8  Comparison of quenching and Proteinase K activation of nanobeacons in different
     buffer conditions.......................................................................................................... 59
Fig. 5.9  Detergent effect on nanobeacon can be demonstrated with Cremophor ...................... 60
Fig. 5.10 Detergent effect can be demonstrated with 1:30 nanobeacon .................................. 61
Fig. 5.11 Comparing the effect of Tween and Proteinase K activation of different
     nanobeacons.................................................................................................................. 62
Fig. 5.12  MMP-7 activation of 1:10 nanobeacon ................................................................. 63
Fig. 5.13  QD alone control ................................................................................................. 64
Fig. 5.14  Tween cleavage control..................................................................................... 65
Fig. 5.15  BHQ1 stability against Tween 80 ..................................................................... 65
Fig. 5.16  Characterization of proteinase K enzyme velocity in different buffers .......... 66
Fig. 5.17  Peptide bond geometry as solved by crystallography .................................... 67
Fig. 5.18  Experimental FRET data matches well with theory ........................................ 69
Fig. 5.19  Michaelis-Menten cleavage simulation $[S] << k_m$ ........................................... 71
Fig. 5.20  Experimental data supporting the theoretical simulation in Fig. 5.19 .......... 72
Fig. 5.21  Michaelis-Menten cleavage simulation $[S] ~ k_m$ .............................................. 73
Fig. 5.22  Michaelis-Menten cleavage simulation $[S] >> k_m$ ............................................ 74
Fig. 5.23  Comparison of MMP-7 activity in tetraborate and MMP buffer .................... 75
Fig. 5.24  Comparison of cleavage experiment with theoretical simulation using literature kinetics parameters ........................................................................................................... 76
Fig. 5.25  Simulation of nanobeacon cleavage following Model 2 in the case when $[S] << k_m$ . 78
Fig. 5.26  Theoretical curve of nanobeacon fluorescence as a function of number of quenchers predicted by Förster analysis ........................................................................................................... 79
Fig. 5.27  Michaelis-Menten simulation of nanobeacon cleavage .................................. 80
Fig. 6.1  QD-Rhodamine Nanobeacon ............................................................................. 84
Fig. 6.2  QD-Rhodamine fluorescence with and without covalent conjugation .......... 85
Fig. 6.3  Chromatographic characterization of 1:5 QD:Rho nanobeacon ...................... 86
Fig. 6.4  Fluorescence imaging of 1:5 QD:Rho nanobeacon ........................................... 87
Fig. 6.5  QD-Rho fluorescence of 1:20 nanobeacon synthesized by covalent conjugation and electrostatic self-assymby .................................................................................. 87
Fig. 6.6  Spectral overlap of QD and Rhodamine Red ..................................................... 88
Fig. 6.7  Cleavage of Rho-GPLGLARK ........................................................................... 89
Fig. 6.8  Proteinase K cleavage of 1:5 QD:Rho nanobeacon .......................................... 90
Fig. 6.9  HPLC data shows complete dissociation of Rhodamines from QDs after cleavage ..... 91
Fig. 6.10  Cleavage kinetics difference between covalently-linked and self-assembled nanobeacons ........................................................................................................................................... 92
Fig. 6.11  MMP-7 activity positive control ....................................................................... 94
Fig. 6.12  MMP-7 cleavage of self-assembled 1:20 QD:Rho nanobeacon ....................... 95
Fig. 6.13  MMP-7 cleavage of covalently-conjugated 1:20 QD-Rho nanobeacon....................... 96
Fig. 6.14  Negative control of 1:20 QD-Rho nanobeacon cleavage ........................................ 96
Fig. 6.15  Hydrophobicity characterization of BHQ1 and Rhodamine.. ................................. 99
Fig. 6.16  Proteolytic nomenclature of amino acid position in a cleavable peptide. ............... 100
List of Appendices

Appendix A  Solubility of Rho-GPLGLARK
Appendix B  QD Quantum Yield Measurement
Appendix C  QD Fluorescence Standard Curve
Appendix D  Failed nanobeacon cleavage conditions
Appendix E  Proteinase K quenching of QD
Appendix F  Tween does not cleave peptide
Appendix G  Sample Michaelis-Menten simulation
Chapter 1
Introduction

Over the past decade, semiconductor quantum dots (QDs) have garnered substantial attention from the biological research and medical communities [1-3] due to their unprecedented multiplexing capability, tunable emission over the entire visible and near-infrared (NIR) wavelength range, superior chemical and photo-stability, bright emission, and versatile surface chemistry. These advantages have led to the utilization of QDs in numerous applications, including immunoassays [4-7], protease assays [8-13], nucleotide assays [14-18], cell labeling [19-21], and in vivo imaging [22-24].

In particular, the promising prospects of QDs for in vivo imaging present key opportunities for using this technology in cancer detection and diagnosis. Recent advances in the molecular understanding of cancer have placed nanotechnology as a frontrunner in translating this knowledge into clinical impact [25]. The opportune confluence of length scales in medicine and technology makes feasible more sophisticated treatment and interaction with tumours at the cellular and molecular level. In vivo imaging, specifically the development of contrast agents and strategies for the early detection and molecular diagnosis of cancer, has been identified as one of the key cancer nanotechnology areas by the US National Cancer Institute [25].

QDs provide an attractive platform for integration into multi-functional imaging nanoparticles because both their optical and surface properties can be customized. Molecular targeting of QDs, particularly in tumor imaging, has been focused on antibody recognition of cell surface markers [26]. A different approach to achieving targeting has been demonstrated in
molecular proteolytic beacons, which can be applied to QDs. Our group has previously reported such a construct based on the peptide GPLGLARK with Pyropheophorbide (Pyro) and Black Hole Quencher-3 (BHQ3) on its two ends [27]. Pyro is quenched by BHQ3 through fluorescence resonance energy transfer (FRET), and the beacon is inactive until specifically cleaved by matrix metalloproteinase-7 (MMP-7). MMP-7 is an extracellular target known to be over-expressed by many cancers [28]. Upon activation, Pyro simultaneously emits NIR fluorescence for imaging and generates singlet oxygen for photodynamic therapy [27]. Selectivity is achieved through specific activation by the target, and relies less on preferential accumulation. Extending upon this work, the same strategy can be applied to QDs to create an MMP-7-sensitive nanobeacon for cancer detection imaging.

In addition to being attractive fluorophores as previously mentioned, QDs have properties that make them ideal FRET donors. For example, their large Stokes Shift minimizes direct excitation of the energy acceptor, their tunable emission allows one to maximize spectral overlap and energy transfer, and their large absorption cross-section efficiently utilizes the energy from the excitation light. Furthermore, beyond the inherent optical advantages of QDs, they can be conjugated to multiple functional moieties by virtue of being nanoparticles [29]. This feature gives nanobeacons unique properties that distinguish them from molecular beacons in two major ways. First, nanobeacons can be functionally expanded in iterative generations. Recent advances in protease-mediated cellular uptake [30, 31], cell surface marker targeting [23], protease-actuated nanoparticle assembly [32], and multiplexed targeting of multiple members of the MMP family [33] are just a few of the possible enhancements that may be incorporated into future nanobeacon designs. Secondly, because multiple quenchers can be attached to a single QD, nanobeacons activation is potentially non-linear and tunable.
It is well known that QD quenching is a non-linear function of both QD-quencher ratio and QD-quencher distance as described by the following equation,

$$E = \frac{nR_0^6}{nR_0^6 + r^6}$$  \hspace{1cm} (1-1) [12]

Where $E$ is energy transfer or quenching efficiency, $n$ is the number of acceptors per donor, $R_o$ is the characteristic Förster distance for a particular donor-acceptor pair, and $r$ is the donor-acceptor distance. Fig. 1.1 presents the relationships captured in equation (1-1).

![Fig. 1.1](image.png)

**Fig. 1.1** Theoretical plot of energy transfer efficiency as a function of quenchers per QD. Each curve represents a different QD-quencher distance. $R_o$ is assumed to be 3.7 nm for this analysis, which is typical for QD-molecular quencher FRET. It is hypothesized that these quenching curves translate into activation curves of nanobeacons.

The property of non-linear quenching is anticipated to translate into non-linear activation (Fig. 1.2), which amplifies the difference in MMP expression levels between healthy and malignant tissues in terms of fluorescence signal. For example, a two-fold difference in MMP expression between tumorous and healthy site can translate into 40 and 20 cleaved quenchers,
respectively, at a presumed time point. Assuming that the nanobeacon was designed with $r=3$ and $n=40$, non-linear activation would result in a fluorescence signal of more than 70 fold difference between the two sites even though the MMP expression difference is only two-fold (Fig. 1.2). Furthermore, the above example also highlights the possibility to tune activation curves by engineering the peptide conformation and QD-quencher ratio. These unique features accessible only to the nanobeacon have significant clinical implication in minimizing false detection because they offer control mechanisms to match probe sensitivity to clinically relevant levels of MMP expression.

![Graph](image)

**Fig. 1.2** Hypothesized nanobeacon fluorescence activation curves. Each curve represents a different QD-quencher distance. $R_0$ is assumed to be 3.7 nm and each nanobeacon is assumed to have 40 quenchers for this analysis.

While several reports exist in the literature on QD FRET protease probes [8-13], a gap remains in designing a nanobeacon for *in vivo* imaging. Specifically, poor colloidal stability, quantum yield loss, and large conjugate size represent potential limitations of applying these probes *in vivo*. Most importantly, to date, complete QD fluorescence activation has not yet been
explicitly reported. It stands to reason that there are more challenges than meets the eye in the proteolysis of nanobeacons. Complete or at least substantial activation of the nanobeacons is indispensable for cancer imaging in overcoming the inherently noisy environment \textit{in vivo}, and in taking advantage of the anticipated non-linear fluorescence activation unique to nanobeacons. Therefore, the overall goal of this thesis to 1) design a nan beacon for a clinically significant tumour target, and 2) elucidate factors influencing cleavage efficiency and achieve substantial proteolysis of nanobeacons. More specifically, the individual aims are,

- To design a nanobeacon that is small, stable, and bright
- To synthesize the nanobeacons and demonstrate successful conjugation
- To elucidate issues associated with nanobeacon cleavage
- To demonstrate complete activation of the nanobeacons by MMP-7

\textbf{Fig. 1.3} Nanobeacon concept. The QD fluorescence is silenced by BHQ1s until the peptide linkers are specifically cleaved by MMP-7. A unique feature of the nanobeacon is that multiple quenchers can be attached to a single QD.
Chapter 2
Background

2.1 Quantum Dots

QDs are semiconductor crystals with physical dimensions smaller than the exciton Bohr radius in that particular semiconductor [1]. They are typically composed of atoms from Groups I-VII, II-VI, or III-V on the periodic table.

The physics of QDs not only make them inherently interesting nanostructures to study on the fundamental level, but also confer them unique optical properties inaccessible to traditional molecular fluorophores. Due to the nanometer size regime of QDs, electron-hole pairs inside these nanostructures experience quantum confinement [34]. Similar to electrons in atoms that exhibit quantized energy states and line-width emissions due to confinement by the nucleus, QDs behave as artificial atoms by confining the electron-hole pair distance. When a QD absorbs a quantum of energy larger than its band gap energy, an electron is promoted to a higher energy state, leaving a hole behind. At a later time, the electron returns to the ground state through recombination with the hole, emitting a photon of energy equivalent to that of the band gap. These mechanisms imply that QDs have continuous absorption bands from slightly above their emission energy all the way into the UV range; they also have near-atomically-narrow emission bands limited only by current synthesis techniques [35]. As the QD size changes, the exciton energy states change, which in turn changes the size of the electronic band gap. In this way, the QD emission wavelength is tuned with its size.
In 1998, Chan and Nie [36] and Bruchez et al. [37] made QDs water-soluble for the first time, bringing them into the biomedical research community. Once water-solubilized, the unique optical properties of QDs - continuous absorption, large Stokes Shift, and narrow and customizable emission - make them highly attractive in numerous biological and medical applications. For the first time, highly multiplexed optical labeling system is made possible [38-40]. QDs’ narrow emissions allow the simultaneous utilization of many colours, and their broad absorption and large Stokes Shift greatly simplify instrumentation by requiring only a single excitation source and mitigating spectral cross-talk. In energy transfer applications, such as in studies of protein interaction by FRET, QDs make up a highly convenient library of optical agents due to their tunable emission. In addition, QDs are also much brighter than traditional fluorophores due to their large absorption cross-sections and high quantum yield, and have superior chemical- and photo-stability. These properties make them highly suitable for applications requiring high sensitivity, \textit{in vivo} imaging, and time-tracking of biological processes. Finally, being a nanostructure with a large surface-to-volume ratio, the versatile surface chemistry of QDs further increases their customizability for a wide range of applications. With its amenability to being modified by various conjugates, including antibodies [26], peptides [2], polymers [41, 42], and oligonucleotides [16], QDs are emerging as a highly promising platform for integration into multi-functional nanoprobe.

A summary comparing and contrasting QDs with a molecular fluorophore (Rhodamine Red-X) is presented on the next page.
Table 2.1 A comparison between QD and Rhodamine Red-X

<table>
<thead>
<tr>
<th>Property</th>
<th>QD</th>
<th>Rho</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightness</td>
<td>✔️</td>
<td>✔️</td>
<td>Despite widespread acceptance of QD’s brightness over molecular fluorophores in the literature, personal experience with three different batches of PAA QDs used in this work seems to give Rhodamine Red a slight edge. Specifically, both of QD’s proclaimed high extinction coefficient and quantum yield shy in comparison to those of Rhodamine. This discrepancy may be ascribed to several reasons: 1) QDs can have large batch variability, and the ones used for this work may not have been optimally engineered, 2) the largest QD used in this work has an emission wavelength of 550 nm, but the extinction coefficient is known to increase quickly with size, 3) concentration and extinction coefficient were determined using absorbance alone based on empirical data recorded in reference [43], which may or may not be accurate for the PAA QDs used, and 4) The QD quantum yield is a function of excitation wavelength. In this work, excitation was always done away from the first excitonic peak, which has the highest quantum yield, to achieve a larger Stokes Shift.</td>
</tr>
<tr>
<td>Photostability</td>
<td>✔️</td>
<td></td>
<td>On a fluorescence microscope using 40× objective and mercury arc lamp excitation, QDs can last for up to hours with only slight photobleaching whereas most fluorophores fade away within a few minutes.</td>
</tr>
<tr>
<td>Chemical Stability</td>
<td>✔️</td>
<td></td>
<td>QD fluorescence can be sensitive to buffer condition (e.g. salt, pH, detergent, etc.) to varying degrees depending on the batch of QD. Furthermore, their colloidal stability is also often a concern. In contrast, Rhodamine, although shifts in wavelength more often, has relatively stable fluorescence intensity in a wide range of conditions. It also resists decomposition in 95% TFA for at least up to an hour.</td>
</tr>
<tr>
<td>Multifunction</td>
<td>✔️</td>
<td></td>
<td>The surface of QDs provides a means of attaching multiple different biomolecules to build multifunctional probes. This feature is not available to molecular fluorophores.</td>
</tr>
</tbody>
</table>
| Customizable     | ✔️ |     | Both the optical and surface chemistry of QDs can be customized, not only by changing its size, but also by changing its composition. The fact that QDs can
retain their surface chemistry while tuning emission color is very valuable for building beacon constructs since beacon-enzyme interaction can sometimes change drastically when the chemistry of the dyes changes.

**Multiplexing** ✓

The large Stokes Shift allows multiple colours of QDs to be excited at a single wavelength, making instrumentation simpler for multiplexed detection. However, it should be noted that flow cytometers as sophisticated as being able to acquire 10 different colours using multiple lasers and filters are already commercially available.

**Ease of use** ✓

The myriad of photophysical processes and the difficulty in characterizing QD structures sometimes make QDs complex to work with, especially in applications where quantitative fluorescence intensity is important. In addition to the environmental sensitivity mentioned above, QD fluorescence is also affected by light exposure and temperature. They can undergo both photobrightening and photobleaching, as well as fluorescence activation and inactivation, even as measurements are taking place. In other words, measuring their fluorescence can sometimes change their fluorescence. Many of these processes have different time-dependence, some are reversible while others are not. Still others have partial reversibility. Depending on buffer, QDs can also be more or less sensitive to each of these variables. Similarly, many of the commonly used techniques in synthetic chemistry (e.g. acid treatment, base treatment, spin dialysis, mixing solvents) have the potential to modify QD fluorescence; if the before-and-after fluorescence intensity is important, probe synthesis becomes tedious to characterize. The non-uniformity of QD population also plays an unknown role. Most importantly, each QD sample can contains a certain percentage of non-fluorescent population whose effect is unclear and difficult to account for. Aside from optical complications, the surface chemistry of QDs also needs considerations in experiments. Due to the inability to perform conventional analytical techniques such as NMR or MS on QDs, structurally, much less can be characterized for QDs at the atomic level. For example, it is not known how many layers of PAA are on a single QD, or whether the layers are patchy. The core-shell-coating structure of QDs can also introduce complications. For example, PAA is known to swell depending on ionic strength and pH. Finally, all this complexity is made even more complex by the large batch variation and variable storage stability (e.g. aggregation or polymer crystallization can sometimes be observed after months of storage) that QDs can sometimes have.
Without a doubt, experimental repeats that have significant time delays become more complicated. Despite all this, experience, patience, and well-made QDs can help to minimize many of the complications mentioned, and as QDs become better engineered and various processes better understood in the future, hopefully some of the complications will disappear. In the end, just as a system becomes more complicated when it gets bigger and when there are more interacting parts, the benefits of working at the nanoscale is, at least in some situations, inevitably accompanied by an increase in complexity.
2.2 Poly(acrylic acid) for QD Coating

High quality QDs are synthesized through the organo-metallic route using high-temperature conditions, and capped by a mixture of trioctylphosphine oxide (TOPO) and alkyl amines [1]. The transfer of these organic QDs into the aqueous phase is a necessary step in using them for biological applications. Current methods to achieve QD water-solubility fall largely into three categories: 1) by ligand exchange of a polar molecule (e.g. mercaptoacetic acid or dihydrolipoic acid) with TOPO [36, 44], 2) by incorporation of QDs into silica nanoparticles [37, 45], and 3) by encapsulating QDs in an amphiphilic shell [22].

QDs prepared by methods in the first category often face colloidal stability issues due to desorption of ligands over time [46-49]. Furthermore, commonly used carbodiimide (e.g. EDC) reactions for conjugation have a tendency to aggregate these QDs [14], leaving metal coordination and electrostatic interaction as the only viable conjugation strategies. Of the two, metal coordination is much more widely used; however, this method is often accompanied by a reduction in the QD quantum yield [9], has poor control over conjugation valence, and faces concerns regarding long-term stability. Even though progress has been made in addressing some of the aforementioned issues [46, 49], with one or two exceptions, this class of QDs is not generally suitable as the basis for nanobeacons.

QDs prepared by methods in the second category, though much more stable, are generally too large for FRET applications [14]. Therefore, QDs in the third category, namely those encapsulated in amphiphilic polymers, were chosen as the basis of the nanobeacons in this work. Specifically, QDs were wrapped in poly(acrylic acid) (PAA) 40%-grafted with
octadecylamines. These QDs are stable [41], relatively small (~13 nm, unpublished data), bright (maintaining as high as 92% of the original quantum yield, unpublished data), photostable [50], and amenable to carbodiimide conjugation with good valence control (Section 6.1).

Compared to another highly successful system consisting of dihydrolipoic acid (DHLA) QDs [51] and His₆ tag [52] conjugation used routinely by Mattoussi et al. to build QD FRET probes [53-56], a system utilizing PAA QDs have some distinct differences. First, PAA QDs are expected to have better colloidal stability than DHLA QDs. Even though no study has been published to date, in theory, DHLA desorbs much less from the QD surface compared to monodentate ligands (e.g. mercaptoacetic acid) that have been used in the past because it is a bidentate ligand [49]. In the same logic, PAA, which wraps around QDs by a large number of hydrophobic interactions, is likely even more stable than DHLA. Secondly, DHLA QDs are known to allow access to the metal surface of QDs [57]. For applications where metal toxicity is a concern [58], PAA QDs have the conceptual advantage of providing better encapsulation [59]. Finally, PAA QDs have been demonstrated to successfully undergo covalent conjugation using carbodiimide chemistry [22]. This provides a more stable conjugation scheme compared to metal coordination. The potential advantages of PAA QDs come at the cost of slightly larger probe size compared to DHLA QDs. This makes them less favourable in terms of energy transfer due to increased donor-acceptor distance, but perhaps more favourable in terms of accommodating multiple functional moieties in the future due to increased surface area.
2.3 Matrix Metalloproteinase-7 as a Cancer Target

Cancer is one of the leading causes of premature death in Canada [60]. According to estimates by the National Cancer Institute of Canada, 39% of women and 44% of men among Canadians will develop cancer in their lifetimes, and approximate 1 in 4 of all Canadians will die from Cancer [60]. Currently, 1 in 38 lives with the disease, requiring treatment and continued care and support [60]. As a disease with escalating social and economic cost and unsatisfactory patient outcomes, cancer has been an intense field of research.

One key area of research for improving cancer survival rate is in early detection [25]. A hallmark of cancer is the uncontrolled proliferation of diseased cells [61]. At a more advanced stage, cancer cells can also intravasate into blood vessels and extravasate to invade another tissue in a process called metastasis. At the root of cancer are genetic mutations, which continue to accumulate throughout the development of the disease. Being able to detect cancer before the formation of visible tumours not only means the disease can be treated before it grows to compromise the surrounding healthy tissues, but also means that the cancer cells are likely to carry fewer mutations [61], which improves the success rate of treatments. The ability to identify cancer at such an early phase will likely depend on high-sensitivity detection of molecular targets expressed by cancer cells, including cell surface markers, mRNAs, and secreted proteins.

In recent years, matrix metalloproteinases (MMPs) have been elucidated as an important player in tumorigenesis [28]. There are a total of 25 known MMPs in human, each can be either soluble or membrane-associated [62]. All of them function at neutral pH, and share both
conserved domains and variable domains for substrate specificity. The proteolytic activities of MMPs against the extracellular matrix (ECM) have been implicated as key to cancer invasion and metastasis [62]. Specifically, MMPs are involved in intravasation, extravasation, local migration, angiogenesis, and the maintenance of tumour microenvironment. In general, the over-expression of MMPs correlates with tumour aggressiveness and poor prognosis [62].

MMP-7, otherwise known as matrilysin, has unique implications in cancer detection among the MMP family because it is one of only few that are expressed by tumour cells themselves rather than by stromal cells [62]. Matrilysin is known to be over-expressed in invasive cancers of the esophagus, stomach, colon, liver, pancreas, lung, skin, breast, prostate, and head and neck [62]. In addition to degrading ECM like other MMPs, matrilysin further promotes tumour invasion and migration by activating proMMP-2 and proMMP-9, and cleaving E-cadherin proteins that function to adhere two adjacent cells [62]. Matrilysin is also one of several MMPs that have recently come to light as important contributors in ectodomain shedding [62]. Shedding refers to the cleavage of transmembrane proteins into soluble products, thereby altering cell signaling and cell-tissue interactions. MMP-7 has been reported to shed Tumour Necrosis Factor α (TNF-α), Insulin-like Growth Factor (IGF), Heparin-binding EGF-like Growth Factor (HB-EGF), Fas Ligand (FasL), E-cadherin, and Tumour Growth Factor β (TGF-β), contributing to a vast number of cancer processes at all stages of disease development, including inflammation, invasion, proliferation, apoptosis evasion, angiogenesis, and metastasis [62].

MMP-7’s involvement in a large number of cancer processes and its correlation with unfavourable prognosis makes it an attractive molecular target for cancer detection and therapy.
Furthermore, as a secreted extracellular target, MMP-7 circumvents the challenging requirements of intracellular probe delivery and endosomal escape. The large size of the MMP family and their closely related functions and roles in tumorigenesis present an exciting opportunity to benefit from the unparalleled multiplexed detection capability of QDs. These characteristics make MMP-7 an apposite model system for the demonstration of QD targeting in the form of a proteolytic nanobeacon.
2.4 Potential False Positives

The specificity of a proteolytic probe is largely conferred by the design of its peptide linker sequence [63]. The sequence chosen for this project (GPLGLARK) was modified from a commercial substrate designed for generic MMP assays [64] (OmniMMP, Biomol International), which is cleavable by multiple members of the MMP family. Even so, it was previously shown, at least in one configuration of dye and quencher, that the sequence is specific to MMP-7 and stable even against the closely related MMP-2 [27]. While this provides some confidence in the selectivity of the peptide sequence used, whether the result can be extrapolated to predict \textit{in vivo} selectivity remains in question due to the large variety of proteases in human and the complexity of the \textit{in vivo} environment. There are a total of 699 known proteases in man [65], many of which operate optimally under different environments and each has a different bio-distribution. This makes it impractical to exhaustively evaluate the selectivity of a peptide sequence. Furthermore, even if selectivity can be verified somehow in theory, MMPs are not exclusively expressed by cancer [66]. Therefore, an assessment of possible false detections is not only prudent, but necessary to accurately convey the potential limitations of imaging cancer by MMP-7 proteolysis.

Aside from the reticulo-endothelial system (RES) that non-specifically uptakes and metabolizes foreign compounds, three likely sources of false positives are considered here: 1) the regular expression of MMP-7 in healthy tissues, 2) the upregulation of MMPs in non-cancerous pathological processes, and 3) the various proteases in human, particularly those circulating in blood. While the most accurate method of carrying out this assessment would be to quantitatively consider the activity levels of all proteases in all human tissues under normal and pathological conditions, this information is not readily available in the literature, likely due to the
lack of a high-throughput method for making these measurements. Instead, a qualitative account is given here based on what is known in the literature of the physiology of different proteases and transcriptome data.

Although MMP-7 has important physiological functions, its expression appears to be largely transient and, for the most part, is kept at relatively low levels in healthy adults. As elucidated by studies in mice and by its homology with other MMPs, MMP-7 is believed to play important roles in the innate immune system and in tissue repair and remodeling [67, 68]. Gene expression studies have found that only lymphoblasts, salivary glands, and pancreatic islets express relatively higher levels of MMP-7 compared to other tissues in human [69]. Together, these data seem to support the general notion that, in the absence of a tissue injury or ongoing immune response, MMP-7 is not normally abundant in humans. However, the same data also suggests that at least some glandular tissues could be possible false positive candidates, consistent with findings from other methods of measuring expression in the literature [67]. It should be noted that some caution should be taken in the interpretation of tissue gene expressions since no information is given about the eventual destinations of the secreted MMPs, and the expression results can vary depending on the databank used. Lastly, human plasma concentration of MMP-7 is known to be below 3.82 µg/L [70] even for patients with metastasized renal cell carcinoma. This is roughly 400 times less concentrated than the assay conditions used in this work (e.g. Section 5.2, 3 hours to complete cleavage at 1.5 µg/mL MMP-7 concentration); therefore, plasma activation of nanobeacons by MMP-7 is not likely a concern.

Members of the MMP family often have overlapping substrates and functions. For example, MMP-7 is known to have large substrate overlaps with MMP-3 [67]. Therefore, it is
important to consider potential false positives that can be caused by diseases that involve elevated levels of MMP. In addition to being involved in immune response and tissue injury in general, MMPs are also more specifically associated with a number of diseases outside of cancer [66]. They have been implicated in respiratory diseases such as pulmonary fibrosis, asthma, emphysema, and acute respiratory distress syndrome, in both osteo- and rheumatoid-arthritis, and in cardiovascular disorders that include aneurysm, atherosclerotic plaque rupture, and myocardial infarction and left ventricular hypertrophy [66]. In particular, MMP-7 is known to have roles in inflammatory diseases of the lung, and has potential connections to multiple sclerosis, Alzheimer’s disease, and arthrosclerosis [68]. All of the aforementioned disorders are potential false positives when attempting to detect cancer by MMP-7 proteolysis.

Finally, since the envisioned method of administration for the nanobeacon is by systemic injection, a general consideration of the various proteases present in human is important, particularly those in blood serum. Of the 183 different peptidase families in the human degradome, four prominent families account for more than 40% of all human proteases [65]. Of the four, serine peptidases raise the most concern for false detection; Ubiquitin-specific peptidases function intracellularly [65], which pose no risk of causing false negatives; prolyl oligopeptidases cleave substrates whose identities remain largely unknown [65]; and adamalysins, even though they function extracellularly in shedding transmembrane proteins [65], are unlikely to cause false detection since nanoparticles usually cannot enter the interstitial space of healthy tissues easily. The family S1 serine peptidases is the largest family in the human degradome and plays a role in many biochemical processes, including digestion, blood coagulation, fibrinolysis, development, fertilization, apoptosis, and immunity [65]. In particular, those proteases involved in immunity and coagulation and clot degradation processes, such as
thrombin, plasmin, and factor XII, constitute perhaps the largest population of proteases in plasma and pose the highest risk for false detection since they can easily access the administered nanobeacons. Fortunately, these proteases are tightly regulated and mostly inhibited under normal physiological conditions, thus greatly reducing the chance of non-specifically activating the nanobeacons.

Overall, not counting RES, tissue injuries, and diseases that solicit an immune response, this qualitative assessment estimates the most likely false detection candidates for an MMP-7 nanobeacon to be the glandular tissues that constitutively express MMP-7 and any of the diseases that are strongly associated with MMPs in general.

2.5 Förster Resonance Energy Transfer

In the 1940’s, Theodor Förster published a series of papers that significantly contributed to the formulation of the modern theory that bears his name [71]. FRET describes the process in which a chromophore in its excited state non-radiatively transfers this energy to another chromophore in the ground state through long range dipole-dipole interaction [53, 72]. The viable distance for this kind of interaction is usually less than 10 nm. The energy transfer efficiency is sensitive to the spectral overlap between donor emission and acceptor absorption, relative dipole orientation, donor-acceptor separation distance, quantum yield of the donor, and refractive index of the energy transfer medium. Because electronic dipoles are not well understood in QDs, it was not immediately obvious whether they can participate in FRET, and if so, whether the same theoretical formalism would describe the energy transfer process. Mattoussi and co-workers were among the few pioneers who detailed the QD FRET process, and
demonstrated that the Förster formalism can be applied to QD FRET with excellent predictive power [12, 72, 73].

In addition, they have made extensions to the Förster theory to give the governing equations for a system of a single donor transferring energy to multiple acceptors [12],

\[
E = \frac{(F_D - F_{DA})}{F_D} \tag{2-1}
\]

\[
R_0 = \left[8.8 \times 10^{23} \kappa^2 n_D^{-4} Q_D J(\lambda)\right]^{1/6} \text{ Å} \tag{2-2}
\]

\[
J(\lambda) = \frac{\int F_D(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda}{\int F_D(\lambda) d\lambda} \tag{2-3}
\]

\[
r = \left(\frac{n(1 - E)}{E}\right)^{1/6} R_0 \tag{2-4}
\]

\[
E = \frac{nR_0^6}{nR_0^6 + r^6} \tag{2-5}
\]

where \(E\) is energy transfer efficiency, \(F_D\) and \(F_{DA}\) is the fluorescence of the donor with and without acceptor, respectively, \(R_0\) is the Förster distance denoting the D-A separation distance at which \(E=0.50\), \(\kappa^2\) is the dipole orientation factor and assumed to be 2/3, \(n_D\) is the refractive index of the solvent, \(Q_D\) is the quantum yield of the donor, \(J(\lambda)\) is the spectral overlap, \(\varepsilon\) is the molar extinction coefficient of the acceptor, \(\lambda\) is the wavelength in cm, \(r\) is the D-A separation distance, and \(n\) is the number of quenchers around a single donor. It should be noted that traditionally, the D-A separation distance is measured from the dipole centres of the donors and the acceptors. The definition is less clear for QDs because the mechanism of energy transfer is not yet completely understood.
2.6 Recent Advances in Optical Protease Probes

Of the many protease assays available, only a small number is transferable to imaging proteases \textit{in vivo}. Generally, there are two strategies to accomplish this [74]. The first is by using affinity-based probes that incorporate antibodies, antibody fragments, or small peptide ligands, which recognize specific protease proteins; the second is by using the so-called smart probes that produce a signal only upon detection of specific proteases. Since affinity recognition generally performs poorly for MMP-7 (Western blot, unpublished data), only activatable probes are considered here.

To date, protease-activatable probes have only been demonstrated in MRI and optical modalities [74]. In particular, optical methods have been gaining popularity in the last decade due to order-of-magnitude cheaper instrumentation, the ability to image in real-time, lack of radiation, and the versatility and variety in the design and selection of probes. Most of the activatable optical protease probes take advantage of Fluorescence Resonance Energy Transfer (FRET) and de-quenching upon proteolysis by the target protease [74]. The first generation probe of this kind was reported by Weissleder et al. in 1999 [75]. The probe was made up of a carrier molecule with a polylysine backbone grafted with methoxy-polyethylene-glycols. 12 to 14 Cy5.5 dyes were conjugated to each carrier molecule to induce autoquenching. Fluorescence was restored upon cleavage, mostly by lysosomal serine and cysteine proteases. Specificity of this probe can be modified by adding peptide substrates of specific proteases between the carrier molecule and the cyanine dyes.
More recently, *in vivo* imaging of MMP-7 has been investigated. McIntyre et al. [76] reported the *in vivo* detection of MMP-7 activity using a proteolytic beacon constructed by covalently coupling a polyamido amino (PAMAM) dendrimer core with fluorescein-labelled peptides and tetramethylrhodamine (TMR). TMR provided the signal to locate the probes within the animal while self-quenched fluoresceins start to emit once they are cleaved away from the dendrimer by MMP-7. The authors reported up to 17-fold fluorescence increase upon cleavage of the beacon. Selective *in vivo* detection of tumour was demonstrated in a mouse xenograft model. Similarly, our own group has demonstrated *in vivo* targeting of MMP-7 using a photodynamic beacon for selective delivery of photodynamic therapy [27].

At the same time, interest in exploring probes or assays based on QD FRET has increased in recent years. In addition to fundamental studies of QD FRET [73], numerous application-oriented probes have been reported. Medintz et al. designed nanoscale biosensors for maltose [54] and soluble TNT [55], Cady et al. compared QD DNA molecular beacons fitted with different linkages and energy acceptors [15], and several research groups have studied protease-activated QD probes [8-13]. This opens the possibility to build an activatable MMP-7 probe that takes advantage of the unique properties of QDs.

Mattoussi and co-workers designed four different QD protease probes for caspase-1, thrombin, collagenase, and chymotrypsin, and reported physical characterizations of each [12]. They proposed a modular design consisting of a DHLA QD energy donor, a His$_6$ tag for conjugation, a rigid helix spacer made of alpha-amino isobutyric acid, a cleavable peptide sequence, and a quencher/dye attached at the end of the peptide. Each QD is linked to multiple energy acceptors. Cleavage kinetics of each enzyme-substrate pair was characterized within the
Michaelis-Menten (M-M) framework, and the kinetics parameters were reported. Selectivity of the probes were briefly confirmed using cross-reaction assays. Finally, enzyme inhibitor assay was proposed as an application. Inhibitors of thrombin were screened for effectiveness by examining their effects on cleavage velocity. The authors successfully monitored the effect of inhibitors on the proteolytic efficiency of thrombin using the reported QD probe.

West and co-workers designed a QD-based protease beacon by tethering multiple gold nanoparticle quenchers to a single QD by collagenase substrate peptides [8]. Fluorescence data provided evidence for QD quenching by gold and activation by collagenase. Response time of the enzymatic assay was reported to be 47 hours with an observed fluorescence rise of 52%. The relatively long response time was speculated to have been caused by steric hindrance of the nanoparticles and QD-gold aggregation during conjugation.

Shi et al. described a probe with rhodamine-modified RGDC peptides self-assembled on the QD surface through cysteine-metal coordination [9, 10]. They reported a 4-fold decrease in quantum yield upon peptide modification of QD even without rhodamine. Regardless, the emission was sufficiently bright for the quantitative analysis of the FRET probe, and proteolysis was found to be dependent on both trypsin and collagenase concentration. A maximum FRET ratio ($F_d/F_a$) change of 3.5 fold was reported. Both digital fluorescence images and spectroscopic data of the probes after incubation with HTB 126 cells or trypsin solution showed a distinctive colour change. In a similar approach to that taken by Mattoussi and co-workers [12], the authors also demonstrated their FRET probe as a viable platform for screening enzyme inhibitors.
Diverging from popular FRET sensors, Rao and co-workers reported a QD probe with protease-modulated cellular uptake [30]. QDs, through streptavidin-biotin interaction, are conjugated to peptides consisting of three modules: a polyarginine cell-penetrating domain, an MMP-2 or MMP-7 cleavable sequence, and a poly(glutamic acid) blocker domain. The cell-delivery function of the polyarginine is blocked until the probe is cleaved, achieving preferential accumulation with MMP targeting. This probe functionality was demonstrated in cell incubation experiments.

The same group also designed probes that took advantage of the energy transfer properties of QDs. In one work, the authors, using a similar streptavidin-biotin conjugation strategy, linked QDs to substrates of β-lactamase modified with Cy5 [13]. The final probe included a spacer and was co-incubated with free biotin during the self-assembly process to reduce steric hindrance. A 5-fold quenching and 4-fold activation upon β-lactamase cleavage was achieved. In another work, the authors took advantage of Bioluminescence Resonance Energy Transfer (BRET) in their QD protease sensor [11]. BRET can be a valuable approach for QD in vivo imaging because it eliminates the need for an excitation light source [77]. The authors devised a protease assay in which a fusion protein consisting of a Renilla luciferase (Luc8), an MMP-2-specific sequence, and a hexahistidine QD-binding domain was incubated with the enzyme solution. QD-COOH and Ni\(^{2+}\) was added to the mixture after a period of time. The His\(_6\) tag binds to the QDs by Ni\(^{2+}\) coordination and protease activity is detected by the resulting BRET efficiency. A cleaved fusion protein would exhibit weak or no BRET while an intact fusion protein would exhibit strong BRET. The authors reported a maximum BRET ratio (\(F_a/F_d\)) change from 1.64 to 0.21 within 2 hours of assay time, and a sensitivity of 2 ng/mL (30 pM) using a 10% BRET ratio change criterion. Previously reported FRET sensors have
sensitivities in the range of 0.5 µg/mL. However, the heightened sensitivity may or may not be directly attributed to BRET because other factors such as the specific protease-substrate system chosen and the pre-incubation in this 2-step assay can also play a role.

With one exception, the QD probes reported thus far have been designed in view of protease and inhibitor screening assays. In vivo detection and imaging of protease activity poses unique challenges to overcome. Due to the inherently noisy in vivo environment [22], the probe should be designed to have bright emission, a large signal difference pre- and post-proteolysis, fast response time, and appropriate sensitivity. In the work by Mattoussi et al., substantial activation of the QD FRET probe was not explicitly reported. Similarly, Shi et al. and West and co-workers observed less than one-fold fluorescence increase upon activation. Furthermore, probes that use ligand exchange for conjugation face reduced QD quantum yield and concerns in colloidal stability. Rao and co-workers were able to restore over 70% of the quenched QD fluorescence in their β-lactamase beacon. However, the increased size of the probe from using streptavidin-biotin chemistry reduces its utility for in vivo applications. Clearly, there is still more work to be done in designing an optimal in vivo nanobeacon.

Nevertheless, the timely advent of advances in both QD nanobeacon designs and in vivo MMP-7 detection present an exciting opportunity to specifically design an in vivo nanobeacon that can take full advantage of the unique properties of QDs in cancer detection imaging.
Chapter 3
Materials and Methods

3.1 Materials

Black Hole Quencher-1 (BHQ1)

Black Hole Quencher-1 was chosen due to its broad absorption spectrum (Fig. 3.1), reasonable extinction coefficient ($34000 \text{ M}^{-1}\cdot\text{cm}^{-1}$), and lack of native fluorescence.

![Absorption Spectrum and Structure of Black Hole Quencher-1](image)

**Fig. 3.1** Absorption spectrum and structure of Black Hole Quencher-1.
Rhodamine Red-X

Rhodamine was chosen as an energy acceptor because it has a large extinction coefficient (129,000 M\(^{-1}\).cm\(^{-1}\)), absorption in a region that is most reproducible for QD synthesis, and emission that is visible under the microscope (Fig. 3.2).

![Graph showing absorption and emission spectra of Rhodamine Red-X.](image-url)

*Fig. 3.2* Absorption and emission spectra and structure of Rhodamine Red-X.
**Tween 80**
- Molecular weight 1310 Da
- Non-ionic surfactant
- Critical micelle concentration 0.012 mM [78]

![Structure of Tween 80](image)

**Cremophor EL**
- 35 moles of ethylene oxide reacted with 1 mole of castor oil
- Mixed product with the main component being polyethylene glycol esters
- Non-ionic surfactant
- Molecular weight about 2500 Da [79]
- Critical micelle concentration 0.009 wt/vol% [80]

**MMP Cleavage Assay Buffer**
- pH 7.47
- 50 mM HEPES
- 10 mM CaCl$_2$
- 10 mM ZnCl$_2$
- 0.05% Brij-35 (non-ionic detergent Polyoxyethylene glycol dodecyl ether)
- NaOH

**MMP-7 Storage Buffer**
- 50 mM Tris
- 5 mM CaCl$_2$
- 300 mM NaCl
- 20 µM ZnCl$_2$
- 0.5% Brij-35
- 30% v/v glycerol

**Proteinase K Buffer**
- pH 8.0
- 50 mM Tris-HCl
- 5 mM CaCl$_2$
- 50% v/v glycerol

**Borate Buffer**
- pH 8.5
- 10 mM sodium tetraborate
- HCl

**OmniMMP Fluorogenic Substrate [81]**
- Sequence: Mca-PLGL-Dpa-AR
- Compared the sequence in this work GPLGLARK (arrow indicates cleavage site)
- Mca=(7-methoxycoumarin-4-yl)acetyl
- Dpa=N-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl
- Mca fluorescence normally quenched by Dpa by 99.5%
- Dissolved in DMSO 20mg/mL

**Proteinase K Enzyme**
- Cleaves the COOH side of G, A, V, L, I, Y, W, F
- Applied to nanobeacon BHQ1-G PLLGLAR K-QD
3.2 Nanobeacon Synthesis Protocol

CdSe/ZnS core/shell QDs were synthesized using previously described organometallic hot injection method [82-84]. Organic QDs stabilized by TOPO were water-solubilized by coating them in poly(acrylic acid) 40%-grafted with octadecylamine. Concentration of the resulting QDs can be measured by experimentally-derived extinction coefficients [43].

The peptide GPLGLARK was synthesized using standard fmoc solid state peptide synthesis techniques. Sieber resin and HOBT/HBTU coupling agents were used. Mass of the synthesized product was confirmed by HPLC-MS. Conjugation of BHQ1-COOH or Rho-NHS to the peptide was done in solid phase using the following scheme. The resulting product was purified by repeated precipitation in diethyl ether.

Dye to peptide conjugation scheme in solid state

\[
\begin{align*}
\text{Fmoc-GPLGLAR(pbf)K(Ntt)-resin} & \quad \downarrow (1) \quad 20\% \text{ piperidine in DMF, calculate peptide amount by cleaved Fmoc} \\
\text{NH}_2\text{-GPLGLAR(pbf)K(Ntt)-resin} & \quad \downarrow (2) \quad 3\times \text{excess dye and 10x excess HOBT/HBTU in DMF with 5\% DIPEA overnight, wash with DMF} \\
\text{Dye- GPLGLAR(pbf)K(Ntt)-resin} & \quad \downarrow (3) \quad 95\% \text{ TFA and 5\% TIS for 1 hr} \\
\text{Dye-GPLGLARK(NH}_2\text{-CONH}_2 & 
\end{align*}
\]

To assemble the final nanobeacon construct, dissolve the dye-peptide in water (or DMSO if the effect of DMSO in future assays is not a concern) and mix with PAA QDs at the desired
ratio in a buffer that does not disrupt electrostatic interactions. The self-assembly is usually complete within minutes.

Covalent QD-Rho nanobeacons were conjugated using carbodiimide chemistry [85]. PAA QDs and Rho-GPLGLARK were mixed at the desired ratio in 33% borate buffer and 66% DMSO with 1000×QD excess EDC coupling reagents. The reaction was allowed to proceed for 2-3 hours before the sample was purified by 10,000 MWCO spin column dialysis and exchanged into borate buffer or pH11 NaOH water.

Protocols for HOBT/HBTU conjugation were followed for QD-BHQ1 nanobeacon, but the nanobeacon was shown to be conjugated by self-assembly with no covalent linkage (Fig. 5.3). Desired ratios of PAA QDs and BHQ-GPLGLARK were mixed in 33% water and 66% DMSO with 1.5×peptide excess HOBT/HBTU coupling agents and 10% DIPEA. The reaction was run overnight before the sample was purified by 10,000 MWCO spin column dialysis and exchanged into pH11 NaOH. Presumably, the self-assembly process occurred during buffer exchange.

3.3 HPLC Characterization Methods

Two main HPLC methods were used in this thesis. Method 1 uses size exclusion chromatography to separate QDs from free peptides. Method 2 uses reversed-phase chromatography to characterize hydrophobicity of peptides.
**Method 1**

Column: Tosoh Bioscience TSKgel G4000SWxl size exclusion column  
Mobile Phase:  
A) 1 mM phosphate buffer  
B) Acetonitrile (ACN)  
C) 0.1% trifluoroacetic acid (TFA)  
D) Distilled water

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase (%)</th>
<th>Flow rate (mL/min)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 0 0 100</td>
<td>0.5</td>
<td>Initial condition</td>
</tr>
<tr>
<td>20</td>
<td>0 0 0 100</td>
<td>0.5</td>
<td>Water to elute QDs</td>
</tr>
<tr>
<td>21</td>
<td>0 0 0 100</td>
<td>0.25</td>
<td>Slow down flow for better phase transition</td>
</tr>
<tr>
<td>22</td>
<td>0 45 55 0</td>
<td>0.25</td>
<td>ACN / 0.1% TFA to elute peptides</td>
</tr>
<tr>
<td>23</td>
<td>0 45 55 0</td>
<td>0.5</td>
<td>Speed up flow to normal rate</td>
</tr>
<tr>
<td>60</td>
<td>0 45 55 0</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>100 0 0 0</td>
<td>0.5</td>
<td>Phosphate buffer to regenerate column modified by ACN/0.1% TFA</td>
</tr>
<tr>
<td>170</td>
<td>100 0 0 0</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>0 0 0 100</td>
<td>0.5</td>
<td>Equilibrate column with water</td>
</tr>
<tr>
<td>200</td>
<td>0 0 0 100</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

Because the column is packed with silica beads with negative charges, peptides that are self-assembled on the QD surface by electrostatic interaction can dissociate during chromatography.

**Method 2**

Column: Agilent Zorbax 300SB - C18 × 250 mm reversed phase column  
Mobile Phase:  
B) Acetonitrile (ACN)  
C) 0.1% trifluoroacetic acid (TFA)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase (%)</th>
<th>Flow rate (mL/min)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 20 80 0</td>
<td>0.4</td>
<td>Gradient from 20/80 ACN/0.1%TFA to pure ACN, hydrophilic molecules elute first</td>
</tr>
<tr>
<td>40</td>
<td>0 100 0 0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>0 100 0 0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>0 20 80 0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0 20 80 0</td>
<td>0.4</td>
<td>Column equilibration</td>
</tr>
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</table>
Chapter 4
Electrostatic Self-Assembly of QD-Peptide Bioconjugates

Electrostatic self-assembly is the main conjugation strategy used for the synthesis of the nanobeacons in this work. This chapter explains the rationale and the benefits of the strategy, and investigates the stability of the bioconjugate under various buffer conditions.

4.1 Introduction

There currently exist three broad categories [14, 86, 87] of conjugation strategies in forming QD-based bioconjugates for biomedical applications: 1) covalent conjugation involving the formation of amide or disulfide bonds on the surface coating of QDs, most commonly through carbodiimide or thiol chemistry, 2) direct metal coordination to the QD surface through thiol or polyhistidine moieties, and 3) non-covalent conjugation through biotin-avidin, electrostatic, or hydrophobic interactions.

Each strategy offers a different set of advantages and shortcomings. Covalent conjugation forms the most stable end product, but often requires tedious optimization of the reaction for each specific biomolecule. In addition, purification adds extra complexity to the protocol, and QDs have a tendency to aggregate during reaction [88]. Depending on the yield of the reaction, valence control of this method can also be poor. The second conjugation strategy, metal coordination, is much simpler to perform experimentally. In general, biomolecules with thiols or cysteins are mixed with QDs to displace the organic ligands on their surface, thus simultaneously water-solubilizing and bioconjugating to the QDs [10]. However, this process
uses large amounts of biomolecules, and the final product still requires purification. Poor valence control remains a problem, and the technique is frequently accompanied by a reduction in QD quantum yield and colloidal stability. Furthermore, since the metal surface of the QDs continues to be accessible, more caution is required for applications where metal toxicity is a concern. Methods in the third category, while similar to metal coordination as self-assembly processes, distinguish themselves with stronger binding constants. The positive impact of this is that the conjugation techniques are one-step with virtually 100% yield, require no purification, and provide high precision valence control. Of these methods, biotin-avidin interaction forms the most stable bioconjugate, and is popular due to commercially available streptavidin-coated QDs and a good selection of biotinylated biomolecules. Their main drawback is that the large resulting probe size limits the utility of the probes in FRET and biological applications [14]. Electrostatic interaction has been extensively verified by Mattoussi’s group and others to be an attractive conjugation strategy with little demonstrated drawbacks [88-93], and was shown to be suitable for in vitro applications [91]. The caveat of the technique lies in the careful recombinant protein engineering required when appending charged domains to functional proteins [88]. Finally, hydrophobic interaction is rarely utilized alone because it requires QDs with partially hydrophobic ligands, and has not yet been studied or demonstrated extensively. More frequently, hydrophobic interaction is suggested to play a supplementary role in systems utilizing electrostatic interaction [88].

Of the reviewed conjugation methods, electrostatic conjugation offers many attractive features for QD-peptide bioconjugates, which are the main interest of this thesis. Even though electrostatic self-assembly is a widely accepted technique when used with recombinant proteins, its use with peptides remains unexplored to date. Unlike proteins, peptides are chemically
synthesized. The addition of a charged region into a peptide is easily done during synthesis. This means that with peptides, the main barrier that has thus far prevented the wider utilization of electrostatic conjugation - namely the expertise and time required to engineer recombinant proteins - is completely sidestepped. Furthermore, adding the same charged region to a peptide would result in a much higher overall charge density than if the same were done to a protein, which translates into stronger QD binding. As the field of peptide science advances and the functions of peptides become ever more versatile, finding an optimized strategy for forming QD-peptide bioconjugates becomes increasingly important. Beyond the aforementioned advantages such as ease of synthesis, valence control, and small conjugate size, using electrostatic binding can also dramatically accelerate probe development and optimization. The simplicity of self-assembly allows researchers to quickly synthesize and examine many different probes, such as probes with varying conjugate valences. In the development of multifunctional probes, the anticipated need to optimize ratios of different functional peptides and to understand their cross-interference translates into an immense challenge of having to investigate a formidable number of design permutations. The ability to easily conjugate different peptides in different orders and ratios may be the answer to finally make this problem tractable. Clearly, the unique combination of advantages offered by electrostatic self-assembly makes it a highly attractive conjugation strategy for building QD-peptide bioconjugates.

In recent years, His$_6$ tag has emerged as a highly successful scheme for QD-peptide conjugation [12, 94], among conjugation with other biomolecules [14, 52, 95, 96]. Even though it is a metal coordination technique, polyhistidine tags have avoided many of the disadvantages faced by other schemes in this category due to its strong binding to QD surface [57]. Briefly, peptides with His$_6$ tags are mixed with DHLA QDs and the final conjugates are self-assembled
within minutes. His<sub>6</sub> tag binding shares many features with electrostatic binding, and touts almost all of the same advantages. Nevertheless, the two strategies differ in subtle but distinct ways. Compared to His<sub>6</sub> tags, the use of charged tags (e.g. polylysine or polyarginine) in electrostatic binding can sometimes have deleterious effects on the functions of the peptides. Also, electrostatic binding may be less favourable for FRET applications because the charged tags interact with the QD coating whereas His<sub>6</sub> tags interact directly with the QD surface, which amounts to a difference in QD-peptide separation distance. Conversely, His<sub>6</sub> tags have their own shortcomings when compared to electrostatic conjugation. First, it remains in question whether His<sub>6</sub> tags are immune to all the disadvantages of metal coordination, namely that of reduction in QD quantum yield and colloidal stability, especially as the conjugate valence increases. Secondly, His<sub>6</sub> tags require access to the metal surface of QDs. Not only is the selection of commercial QDs that satisfy this requirement extremely limited, but it also means that the long-term metal toxicity of the resulting bioconjugates remains a concern. Thirdly, even though His<sub>6</sub> tags are conceptually better for FRET applications, the potential need for a large spacer to avoid steric hindrance [12] from the QD ligands may nullify this advantage. Finally, for in vivo applications, covalent conjugation will probably ultimately provide the best solution for stability and biocompatibility reasons. Electrostatic self-assembly lends a natural path to covalent chemistry post-optimization when a lysine is used in the binding tag. Moreover, electrostatic interaction is expected to help overcome some of the limitations of covalent chemistry by greatly improving yield. In the end, electrostatic and His<sub>6</sub> tag conjugation appear to be two equally compelling alternatives for forming QD-peptide bioconjugates. Selection of one strategy over another would depend largely on the intended application and probe design details.
4.2 Characterization of Binding Constant

Determining the stability of bioconjugates formed by electrostatic self-assembly is crucial in evaluating the eventual usefulness of this conjugation method. Measuring the binding constant in buffer condition represents the first step in this assessment. A quantitative characterization would supplement the numerous demonstrations of this technique in the literature to better predict its limitations and suitability in various applications. The model system used in this series of studies is the self-assembly of PAA QD with Rho-GPLGLARK-CONH$_2$ (Peptide$^{2+}$) or Rho-GPLGLARK(COCH$_3$)-CONH$_2$ (Peptide$^{1+}$), each consisting of 2 or 1 positive charges, respectively.

Qualitatively, Peptide$^{2+}$ associates strongly with QDs. The binding remains stable even under electrophoresis conditions with an applied electric field of about 500 V/m (Fig. 4.1).

Fig. 4.1 Gel electrophoresis characterization of electrostatic binding. The lanes are (1) Peptide$^{2+}$ in 50/50 H$_2$O/DMSO (2) Peptide$^{2+}$ in H$_2$O (3) Electrostatically self-assembled QD-Peptide$^{2+}$ and (4) Covalently linked QD-Peptide$^{2+}$. 1.5% agarose gel used.
Quantitatively, the binding constant between PAA QD550 and Peptide$^{1+}$ was measured by titrating peptides in DMSO into a 1 mL cuvette containing varying concentrations of QD. Quenched QD fluorescence was measured and recorded as a percentage of the unquenched QD fluorescence (Fig. 4.2). At high QD concentrations, virtually all of the added peptides bind to the QDs. Therefore, the data points all fall on the same curve (when QD >15 µM). As the QD concentration approaches the dissociation constant $K_d$, a fraction of the added peptides remains free in solution. The fraction of peptides bound becomes less and less as the QD concentration becomes more dilute. This fraction can be monitored using FRET, and is observed as reduced quenching at fixed peptide-QD ratios as the QD concentration decreases.

The total volume of DMSO added varied between 0.2 µL to 20 µL. For QD concentrations below 5 nM, which is in the same order of magnitude as the dissociation constant, no more than 1 µL DMSO was added to minimize DMSO’s effect on hydrophobic interactions.

![Fig. 4.2](image)

**Fig. 4.2** Measuring QD-Peptide$^{1+}$ binding constant by monitoring FRET efficiency. As QD concentration decreases, QD quenching is reduced, implying lower peptide binding percentage.
It is known that for a QD-peptide self-assembly process, the distribution of peptide-QD valences (n) for any given nominal valence N (the average peptide-QD ratio used in reagent mixing) follows the Poisson distribution,

$$p(N, n) = \frac{N^n \exp(-N)}{n!} \quad (4-1) \quad [97]$$

and the energy transfer efficiency, after taking into account this heterogeneity, is given by

$$E(N) = \sum_{n=0}^{N_{\text{sat}}} p(N, n)E(n) \quad (4-2) \quad [97]$$

where $N_{\text{sat}}$ is the maximum number of peptides that can bind to a single QD, and $E(n)$ is given by equation (2-5). The error associated with using the naïve assumption of homogeneity (i.e. all QDs have the same number of bound peptides) decreases as r and N increases [97]. The error is generally less than 10% when $r > R_o$ and $N \geq 4$ [12].

From the raw fluorescence data, the ratio $r/R_o$ is determined to be 1.312 by assuming 100% peptide binding at 50 nM QD concentration and fitting to equation (4-2) (Performed by Matlab). The assumption is reasonable because the same peptide binding curve is essentially followed even when QD concentration decreases to 30 nM or 15 nM (Fig. 4.2). Once $r/R_o$ is determined, it is possible to back-solve for the average number of peptides bound per QD ($N_b$) using the same equation (4-2) for each peptide-QD ratio and QD concentration. The percentage binding can then be obtained by taking the ratio $N_b/N$. Finally, the percentage of peptides bound as a function of QD concentration can be fitted to the binding equation to obtain the binding constant,

$$\frac{N_b}{N} = \frac{[\text{Peptide}]_{\text{QD}}}{[\text{Peptide}]_{\text{Total}}} = \frac{K_b[QD]}{1 + K_b[QD]} \quad (4-3) \quad [98]$$

where $K_b$ is the binding constant, the reciprocal of the dissociation constant $K_d$. The subscripts QD and Total denote peptides associated with QDs and total peptides added, respectively.
Fig. 4.3  Percentage peptide binding as a function of QD concentration. Data is presented in both logarithmic (top) and linear (bottom) scale. The percentage is seen to decrease at lower QD concentrations, in the same order of magnitude as the dissociation constant $K_d$. Error bars represent standard deviation of data points from all QD-peptide ratios.

Experimental results fit well with the theoretical model (Fig. 4.3) (non-linear regression performed by SPSS v15.0). The measured dissociation constant is 1.03 nM. As a point of comparison, antibody-antigen dissociation constant is on the order of 10-100 nM [99]. This implies that electrostatic association, at least in borate buffer, is more stable than antibody-antigen interaction. Significant dissociation only occurs when QD concentration is at the 1 nM
range. Rigorously speaking, “binding site” concentration as opposed to QD concentration should be used to calculate $K_{d}$. However, practically speaking, writing $K_{d}$ in terms of QD concentration is more informative because it allows researchers to more readily predict when dissociation becomes a concern. QDs do not have well-defined “binding sites,” and the number can vary depending on the size and type of QD. Furthermore, it can be argued that the comparison of QD-peptide $K_{d}$ to antibody-antigen $K_{d}$ remains valid because even though each individual “binding site” on the QD may not be as stable as a single antibody-antigen interaction, the fact that a single QD has far more “sites” available than a single antibody drives the overall equilibrium towards less dissociation. Nevertheless, despite the argument that writing $K_{d}$ in terms of QD concentration is reasonable, doing so is not without its caveats. Within the theoretical framework, this is equivalent to treating QD as a single large “binding site” that can accommodate multiple substrates. As a result, each peptide that subsequently binds to the QD can be characterized by a slightly different binding constant. In fact, this is the reason for some of the larger errors observed in Fig. 4.3.
QD:Peptide Ratio = 1:2

Bound Peptide Percentage

QD Concentration (nM)

K_d = 0.308 nM

QD:Peptide Ratio = 1:4

Bound Peptide Percentage

QD Concentration (nM)

K_d = 0.429 nM

QD:Peptide Ratio = 1:6

Bound Peptide Percentage

QD Concentration (nM)

K_d = 0.719 nM

QD:Peptide Ratio = 1:8

Bound Peptide Percentage

QD Concentration (nM)

K_d = 0.945 nM

QD:Peptide Ratio = 1:10

Bound Peptide Percentage

QD Concentration (nM)

K_d = 1.200 nM

QD:Peptide Ratio = 1:15

Bound Peptide Percentage

QD Concentration (nM)

K_d = 1.214 nM

K_d = 0.308 nM

K_d = 0.429 nM

K_d = 0.719 nM

K_d = 0.945 nM

K_d = 1.200 nM

K_d = 1.214 nM
Fitting the binding curve for each individual QD-peptide ratio finds the range of
dissociation constants $K_d$ to be between 0.3 nM and 2.1 nM (Fig. 4.4). Binding can be seen to
weaken slightly at higher peptide-to-QD ratios. This makes sense for two reasons: 1) as the
available binding surface on the QD is taken up, the equilibrium is driven towards more
dissociation, and 2) as valence increases, there are more and more positive charges on the QD
surface, which weakens the binding of any subsequent peptides. It should be noted, however,
that the above analysis characterizes the binding event $\text{QD}_0 + \text{N Peptides} \rightarrow \text{QD}_N$, where the
subscript denotes QD conjugate valence. This means that the measured binding constants are, in
fact, averages of constants that characterize a series of $\text{QD}_N + \text{Peptide} \rightarrow \text{QD}_{N+1}$ events. Of more
interest would be to measure each of these binding constants individually. By fitting the
incremental bound peptide percentage (Table 4.1) to equation (4-3), it is possible to obtain values
closer to these constants. Specifically, the newly obtained constants characterize the events $\text{QD}_0
+ 2 \text{ Peptides} \rightarrow \text{QD}_2$, $\text{QD}_2 + 2 \text{ Peptides} \rightarrow \text{QD}_4$, $\text{QD}_{10} + 5 \text{ Peptides} \rightarrow \text{QD}_{15}$ and so on. The
resulting $K_d$ range from 0.3 nM to 9.6 nM, more than a 30 fold difference. This observation suggests that caution should be exercised as conjugate valence increases. Even though a $K_d$ of 9.6 nM is still comparable to the stronger range of antibody-antigen bindings, the interaction can be one or two orders of magnitude weaker when conjugate valence increases into the hundreds.

Table 4.1 Example calculation for total and incremental peptide binding percentage

<table>
<thead>
<tr>
<th>Nominal Peptide per QD (N)</th>
<th>Peptides bound ($N_b$)</th>
<th>Total Bound Percentage</th>
<th>Incremental Bound Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.8211</td>
<td>1.82/2=0.91</td>
<td>(1.82-0)/(2-0)=0.91</td>
</tr>
<tr>
<td>4</td>
<td>3.4650</td>
<td>0.87</td>
<td>(3.47-1.82)/(4-2)=0.82</td>
</tr>
<tr>
<td>6</td>
<td>5.2864</td>
<td>0.88</td>
<td>0.91</td>
</tr>
<tr>
<td>8</td>
<td>6.8890</td>
<td>0.86</td>
<td>0.80</td>
</tr>
<tr>
<td>10</td>
<td>8.3900</td>
<td>0.84</td>
<td>0.75</td>
</tr>
<tr>
<td>15</td>
<td>12.0301</td>
<td>0.80</td>
<td>0.73</td>
</tr>
<tr>
<td>20</td>
<td>13.1206</td>
<td>0.66</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Data from 7 nM QD concentration used for calculations

All the results presented in this section characterize the binding between PAA QD550 and Peptide$^{1+}$. Preliminary data (not included) have shown that the binding for the same QD with Peptide$^{2+}$ is close to 100% at peptide-QD ratios up to 20 even at 1 nM QD concentration. This indicates that increasing the positive charge from 1 to 2 strengthens the binding constant by at least one or two orders of magnitude, if not more. Even stronger binding is expected as the number of charges increases. Together, these results give reasons to be optimistic about the stability of electrostatic binding in QD-peptide bioconjugates.
4.3 Binding Stability Assessment

For interactions that are electrostatic in nature, an important consideration is their salt stability. In addition, literature suggests that hydrophobic interactions can play a supplementary role in determining the stability of these systems [88]. This section explores the forces behind the self-assembly between PAA QDs and the positively charged Peptide$^{2+}$. In particular, salt and detergent stability of the system is studied.

To investigate the effect detergent has on the QD-peptide self-assembly process, different combinations of QD, Peptide$^{2+}$, Tween 80, and Cremophor EL were mixed in different orders to see if detergent disrupts the association as monitored by FRET (Fig. 4.5).

Fig. 4.5 Effect of detergent on QD-peptide self-assembly by fluorescence. Brackets are read as in mathematical expressions. Experiments done in 10 mM tetraborate buffer. Rho denotes Peptide$^{2+}$. Detergent incubation with Peptide$^{2+}$ was over 1 hr, and self-assembly with or without detergent was done overnight. Excitation was done at 450 nm. 0.025 nmol QD and 0.5 nmol of Peptide$^{2+}$ in a total volume of 80 µL were used in all samples. Detergents were used at 0.5%.

Energy transfer between QD and Rhodamine was not disrupted by detergent regardless of the type of detergent used or the order of its addition (pre-self assembly vs. post-self assembly).
In all cases, similar levels of energy transfer were observed. In fact, adding detergent slightly increased energy transfer as seen in the reduction of QD fluorescence and the enhancement of Rhodamine fluorescence in the case of Cremophor addition (Fig. 4.5). Rho+crm was used as control instead of Rho alone due to solubility issue (see Appendix A). Overall, this data shows no evidence of disruption of the QD-peptide electrostatic interaction by detergent.

**Fig. 4.6** Effect of detergent on QD-peptide self-assembly by gel electrophoresis. The lanes are (1) QD alone (2) Peptide$^{2+}$ with Cremophor (3) Peptide$^{2+}$ (4) QD + Peptide$^{2+}$ (5) QD + (Tween + Peptide$^{2+}$) (6) (QD + Peptide$^{2+}$) + Tween (7) QD + (Cremophor + Peptide$^{2+}$) (8) (QD + Peptide$^{2+}$) + Cremophor. Both images show fluorescence data, with the left displaying broad-range visible light under UV excitation captured as a monochromatic image in BioRad GelDoc system, and the right displaying colour digital photograph under UV excitation as taken by a digital camera. The same amount of QD and rhodamine-peptide was added to each well. Electrophoresis was done at 100 V for 15 min.

Gel data shows that detergent does weaken the QD-peptide association, even though not to a significant enough extent to dissociate them in solution (Fig. 4.5, Fig. 4.6). Under electric field, Peptide$^{2+}$ begins to separate from the QD in the presence of detergent (Fig. 4.6 lanes 5-8),
with Tween exercising a stronger effect than Cremophor. Both detergents were added at 0.5 vol% (equivalent to 4.1 mM Tween and 2.1 mM Cremophor), but it is unclear whether the difference in their disruptive ability is a function of detergent molar concentration or detergent chemistry. Whether detergent was added pre-self assembly or post-self assembly made no observable differences (Comparing lanes 5 vs. 6 and 7 vs. 8, Fig. 4.6). In borate buffer, QD-peptide\(^{2+}\) showed no separation even under an electric field (Fig. 4.6, lane 4). Similar to what is observed in solution (Appendix A), Peptide\(^{2+}\) fluoresced more in the presence of Cremophor than without (Comparing lane 2 and 3, Fig. 4.6), presumably due to solubility issues. Overall, the results demonstrate that hydrophobic forces play a role in the QD-peptide self-assembly process, which is in agreement with suggestions in the literature [88].

Next, the stability of the QD-peptide conjugate is studied in different buffers, with a particular focus on salt stability.

**Fig. 4.7** Stability of self-assembled QD-peptide\(^{2+}\) in different buffers. The MMP buffer used consists of 50 mM HEPES, 10 mM CaCl\(_2\), 10 mM ZnCl\(_2\), and 0.05% Brij-35 detergent. The borate buffer used has 10 mM sodium tetraborate. Ratio of peptide to QD was 20. QD concentration was 67 nM in 1 mL total volume. Excitation was done at 450 nm.
Fig. 4.7 shows the stability of QD-Peptide$^{2+}$ electrostatic binding in various buffers, using a covalently linked QD-Peptide$^{2+}$ as a control. The binding is seen to be stable except in MMP buffer. To elucidate which component of the MMP buffer is most responsible for disturbing the electrostatic interaction, PAA QDs and Peptide$^{2+}$ were first dissolved in water, and each component of the buffer was added sequentially in two different orders. The experiment serves to help better understand the roles that each hydrophobic and electrostatic interactions play, and the effect of different salts.

![Graph 1](image1.png)

**Fig. 4.8** Characterizing mechanisms of QD-peptide self-assembly. By adding different salts and detergent in different orders to the QD-peptide conjugate, insights into the roles of electrostatic vs. hydrophobic forces can be gleaned. 67 nM of 1:20 QD:Rho bioconjugate in 1 mL was used. Component injections resulted in less than 30 µL (3%) of total volume increase. Excitation was done at 450 nm.
QD-peptide dissociation was monitored by change in FRET efficiency as each component of the MMP buffer was added. In Fig. 4.8, it can be seen that adding 10 mM of CaCl$_2$ caused partial dissociation. This can be explained by the displacement of peptides from the QD surface by Ca$^{2+}$ ions. Adding ZnCl$_2$ showed no strong evidence of further dissociation. However, once Brij-35 detergent was added, complete dissociation was observed. It can be reasoned that low solubility of the Peptide$^{2+}$ in water (Appendix A) constitutes an energy barrier for the peptides to dissociate from the QD surface. Once this energy barrier is removed, more peptides can be readily displaced by the Ca$^{2+}$ and Zn$^{2+}$ ions.

In Fig. 4.8, it is observed that detergent alone does not dissociate peptides from QDs. This implies that either electrostatic interaction is the dominant driving force in this self-assembly, or that solubility only exerts a synergistic effect when there is a separate, independent mechanism of QD-peptide association. Adding HEPES caused some peptide dissociation, and complete dissociation is seen only after adding CaCl$_2$. Compared to the previous experiment, it can be seen that Ca$^{2+}$ disturbs the electrostatics interaction more when compared to HEPES because Ca$^{2+}$ was able to completely dissociate all peptides in the presence of brij-35 even without HEPES. In addition, it does so at a lower concentration. On the other hand, HEPES in combination with detergent is not sufficient to completely dissociate the peptides. This observation makes sense because Ca$^{2+}$ is divalent while HEPES is a zwitterion.

To further investigate the stability of the electrostatic QD-peptide construct, dissociation at increasing concentrations of NaCl was monitored with or without Brij-35 (0.05%). NaCl was chosen because it is a physiologically relevant salt.
**Fig. 4.9** Salt stability of self-assembled QD-Peptide$^{2+}$. Dissociation of peptide is monitored by a decrease in FRET. Difference in stability is observed with and without detergent. Assay used 67 nM 1:20 QD:peptide$^{2+}$ conjugate in 1 mL borate buffer. Fluorescence was corrected to account for dilution upon addition of NaCl. A total of 225 µL (22.5%) was added to the total volume at the highest salt concentration.

It can be seen that without detergent, only very slight dissociation occurs and only at very high salt concentrations (Fig. 4.9). QD fluorescence activated minimally while Rhodamine
fluorescence appears to be quenched by high salt. With detergent, appreciable amount of
dissociation can be seen above 100 mM salt concentration with complete dissociation being
reached around 500 mM. Overall, the data indicates that peptide binding to QDs through
electrostatic interactions is stable under salt conditions similar to that of the physiological
environment, in the range of hundreds of millimolars.

4.4 Conclusion

Electrostatic binding is an extremely convenient, high-yield, viable method for forming
QD-peptide bioconjugates, thanks to its strong binding and self-assembly nature. The
dissociation constant of the interaction between QD and a single-charge peptide was measured to
be on the order of 0.1 to 1 nM in buffer, comparable to those of antibody-antigen interactions.
When the peptide has two charges, the interaction is stable up to an ionic strength of 800 mM.
This strong observed binding is attributed to the large binding surface of QDs and contribution
from hydrophobic forces. The electrostatic interaction between PAA QDs and GPLGLARK
peptides is deemed sufficiently stable for the studies of this thesis. In the future, the number of
charges on the peptide can be easily increased to produce even more stable conjugates for
physiological environments.
Chapter 5
QD-BHQ1 Nanobeacon

BHQ1-GPLGLARK-QD was the first nanobeacon to be synthesized for this project. Even though protocols for HOBT/HBTU conjugation were followed (Section 3.2), it was later discovered that the peptides were conjugated to the QDs through non-covalent electrostatic self-assembly. The synthesis ratios of QD:BHQ1 ranged from 1:2 to 1:30. Characterization of this nanobeacon elucidated peptide conformation as a key factor in determining cleavage efficiency. Theoretical work in Michaelis-Menten simulation of nanobeacon cleavage progression, which guided the selection of cleavage assay conditions, is also presented.

5.1 Synthesis Characterization

Success of peptide synthesis and subsequent BHQ1 conjugation was confirmed by HPLC, where mass and purity of BHQ1-GPLGLARK was verified (Fig. 5.1).

**Fig. 5.1** Chromatogram of BHQ1-GPLGLARK. Expected mass is 1296.
QD-BHQ1 nanobeacon was conjugated by electrostatic self-assembly. Conjugation was validated by an increase in BHQ1 absorbance and QD quenching as the ratio of BHQ1 to QD increased from 2 to 30 (Fig. 5.2). No free BHQ1 can be observed by eye during purification by either centrifuge membrane filtration devices or dialysis. In addition, size exclusion chromatography confirmed that the nature of the QD-BHQ1 linkage is non-covalent (Fig. 5.3). Overall, evidence suggests that QD-BHQ1 conjugation was successful with good valence control.

**Fig. 5.2** QD-BHQ1 nanobeacon characterized by quenching and absorbance. Quenching and contribution from BHQ1 absorbance can be seen to increase with BHQ1-to-QD ratio. Fluorescence data was normalized against absorbance at excitation wavelength.
Fig. 5.3 QD-BHQ1 nanobeacon characterized by HPLC. Chromatograms from top to bottom show nanbeacons of different QD-BHQ1 ratios. All nanobeacons are shown to be electrostatically self-assembled as each sample contained a large amount of free BHQ1-peptide seen at 56 min elution time. A percentage of the BHQ1-peptides remain associated with the QDs (10.30 min) because their dissociation is caused by silica bead packing competing against the QDs for peptide binding, which is not 100% efficient.

The QD chosen and BHQ1 have good spectral overlap (Fig. 5.4). Even though a new absorption peak at 480 nm (Fig. 5.2) and slight background fluorescence was found with BHQ1 after acid treatment (Section 3.2), the overall quenching scheme is maintained.

Fig. 5.4 Spectral overlap of QD and BHQ1.
5.2 The Effect of Peptide Conformation on Cleavage Efficiency

It was observed in an experiment that the addition of Tween 80 to a mixture of nanobeacons and Proteinase K is apparently able to drastically change the efficiency of cleavage. This led to the formulation of a hypothesis and the subsequent work to support it.

**Hypothesis:** Peptide conformation determines, to a large degree, the cleavage efficiency of a nanobeacon. Surfactants open up the hydrophobic peptide conformation into a freely extended form in water, increasing the likelihood of cleavage either by being a more favorable conformation for enzyme binding or by moving the cleavage site further away from QD to avoid charge repulsion (MMP-7 has pI 5.9 [100]) and steric hindrance.

**Supporting Evidence**

a) Fig. 5.5. Addition of Tween reduces quenching, indicative of an increase in QD-quencher distance, consistent with the opening of peptide conformation. Controls: a, b, c, d

b) Fig. 5.6, Fig. 5.7. Tween is necessary for the activation of nanobeacon by Proteinase K in PBS. Activation is the same regardless of whether Tween was injected first or Proteinase K was injected first. In both cases, near-complete restoration of fluorescence is achieved (after accounting for unexplained Proteinase K quenching). This is consistent with the hypothesis that Tween opens up peptide conformation to allow for enzyme binding. Controls: a, b, c, d

c) Fig. 5.8. The amount of cleavage of a nanobeacon is a function of the amount of quenching. The more quenching, the less cleavage. This is consistent with the
hypothesis that when there is more quenching, the peptide is in a more closed conformation, inhibiting cleavage.

**d)** Fig. 5.8, Fig. 5.9. Brij-35 in MMP-7 buffer and Cremophore, both non-ionic surfactants like Tween 80, reduces quenching of QD nanobeacon. This supports the hypothesis that Tween changes the conformation of the peptide due to a generic detergent effect, not some other Tween-specific effect.

**e)** Fig. 5.10, Fig. 5.11. The effect of Tween in helping cleavage can be similarly observed in 1:2, 1:20 and 1:30 nanobeacons as was observed in the 1:10 nanobeacon. The trend in quenching from 1:2 to 1:30 is conserved post-Tween injection, consistent with the idea that quenching should still increase from 1:2 to 1:30 with an open peptide conformation. All nanobeacons can be substantially activated by Proteinase K in the presence of Tween.

**f)** Section 5.3. Analyzing the data within the Förster theoretical framework shows that the observed quenching change can be accounted for by a D-A distance change less than the peptide length.

**g)** Fig. 5.12. The detergent effect can similarly help MMP-7 cleavage as with Proteinase K.

Note: All experiments used detergent concentrations above their respective CMCs.

**Controls**

**a)** Fig. 5.13. Tween does not influence the fluorescence of QD.

**b)** Fig. 5.14. Tween does not cleave Mca-PLGL-Dpa-AR to any appreciable degree.

**c)** Fig. 5.15. Tween does not decompose or modify the absorbance of BHQ1.

**d)** Fig. 5.16. Tween does not deactivate Proteinase K. Proteinase K has strong activity in the buffer conditions and concentration and substrate-enzyme ratio chosen for the assays. Proteinase K has high activity without Tween.
Fig. 5.5 Effect of Tween 80 on nanobeacon quenching and cleavage. Reduction in quenching is observed upon addition of Tween. Fluorescence signal activation is observed upon addition of Proteinase K. Assay used 0.258 μM of 1:10 nanobeacon (2.58 μM cleavable peptide) in 1 mL PBS. Tween arrows denote 1 μL (0.1%) injections. 50 μL (30.8 μM) of Proteinase K was used for cleavage. Red line indicates unquenched QD alone fluorescence at 0.258 μM as calculated from standard curve (Appendix C).

Experimental result shows that Tween reduces QD quenching (Fig. 5.5), consistent with the hypothesis that increasing peptide solubility unravels its conformation. The first 20 min of the experiment shows a relatively stable nanobeacon fluorescence signal in PBS alone. Then, upon Tween injection, quenching is reduced immediately as well as over time. No dramatic effects were observed upon each subsequent Tween injection. Under the working hypothesis, the explanation is that the hydrophobic peptide-BHQ1s on the nanbeacons slowly turn into a more open conformation due to detergent effects of Tween, thus reducing FRET quenching. The addition of Proteinase K cleaves the peptides, achieving complete restoration of fluorescence. QD is then quenched either by Proteinase K or its buffer, as demonstrated in control (Fig. 5.13).
**Fig. 5.6** Tween is necessary for peptide cleavage. Proteinase K (30.8 \( \mu \text{M} \)) injection has no effect on nanobeacon fluorescence for 76 min until Tween (1 \( \mu \text{L} \)) injection. The explanation is that Proteinase K cannot cleave the peptides due their closed conformation. However, upon Tween injection, the peptides open up and allow cleavage. Assay used 0.258 \( \mu \text{M} \) of 1:10 nanobeacon (2.58 \( \mu \text{M} \) cleavable peptide) in 1 mL PBS.

**Fig. 5.7** Combining data in Fig. 5.5 and Fig. 5.6 shows that the final QD fluorescence is consistent with each other regardless of the order of Proteinase K and Tween injection.
Fig. 5.8 Comparison of quenching and Proteinase K activation of nanobeacons in different buffer conditions. Assay used 0.258 µM 1:10 nanobeacon (2.58 µM peptide) and 30.8 µM Proteinase K. 0.5% Tween 80 was used.

Experimental results show that both QD quenching and the degree of activation is a function of buffer (Fig. 5.8). In PBS, no activation was seen even 70 min after Proteinase K injection. This is consistent with the notion that in an aqueous buffer, the peptides have a closed conformation, which prevents cleavage. In MMP Buffer, fluorescence quenching is slightly decreased compared to when in PBS, and some activation was possible over the course of an hour. It is hypothesized that having Brij-35 (0.05%, a non-ionic surfactant) in the buffer partially opened the conformation of the peptides. This decreased FRET efficiency and allowed some cleavage. An alternative explanation is that only a sub-population of nanobeacons was rendered activatable by the surfactant. However, this scenario is unlikely because the ratio of surfactant to nanobeacon is more than a thousand to one (0.42 mM to 0.26 µM), allowing for enough surfactants to interact with all nanobeacons. Finally, in PBS with Tween, QD quenching was significantly reduced over the course of several hours after Tween injection, after which complete activation was observed with Proteinase K injection. It is hypothesized that Tween
allowed for a highly open conformation of the peptides, strongly decreasing FRET efficiency and allowing complete cleavage. Even though the increase in fluorescence was relatively small, still, a large number of peptides had to have been cleaved off for the observed increase due to low FRET efficiency as a consequence of open peptide conformation (Fig. 1.2).

Fig. 5.9 Detergent effect on nanobeacon can be demonstrated with Cremophor EL. Assay used 0.258 μM of 1:10 nanobeacon (2.58 μM peptide) in 1 mL PBS. 5 μL of Cremophor EL and 30.8 μM of Proteinase K were injected. Red line represents unquenched QD alone fluorescence at 0.258 μM as calculated from standard curve (Appendix C).

Cremophor EL was used to demonstrate that the effect observed with Tween 80 was a generic detergent effect (Fig. 5.9). Cremophor was chosen because it is a non-ionic surfactant like Tween, and is known to be biocompatible for use in drug formulations. When injected at 1 min, a sudden increase in fluorescence was observed. The fluorescence increased slowly over time until Proteinase K injection at 350 min, when complete activation of the nanobeacon was achieved. Even though the specific fluorescence change is different, the overall pattern here is the same as when using Tween, consistent with the hypothesis that the observed effects of Tween are due to its detergent activity.
Fig. 5.10 Detergent effect can be demonstrated with 1:30 nanobeacon. Assay used 0.121 µM of 1:30 nanobeacon (3.63 µM peptide) in 1 mL PBS. 0.5% Tween 80 and 30.8 µM Proteinase K were injected. Red line represents unquenched QD alone fluorescence at 0.121 µM as calculated from standard curve (Appendix C).

Under the working hypothesis, the detergent effect in opening peptide conformation should apply to all nanobeacons, regardless of peptide-to-QD ratio. Indeed, this is what was observed (Fig. 5.10). The first 20 min showed stable nanobeacon fluorescence in PBS. Injection of Tween resulted in a sudden increase in fluorescence. The fluorescence continued to increase slowly over time until Proteinase K was injected at 420 min, which resulted in the complete activation of the nanobeacon. This behaviour is completely analogous to that observed with the 1:10 nanobeacon. More quenching is retained after Tween addition in the 1:30 nanobeacon than in the 1:10 nanobeacon. This is consistent with the hypothesis that Tween exerts its effects by changing peptide conformation because more quenching is expected in the 1:30 beacon even when the peptides are in an extended conformation.
**Fig. 5.11** Comparing the effect of Tween and Proteinase K activation of different nanobeacons. Trend in quenching is preserved even with Tween. Assay used 0.25 μM of each nanobeacon, 0.5% Tween 80, and 30.8 μM Proteinase K in 1 mL PBS. Red line represents unquenched QD alone fluorescence at 0.25 μM as calculated from standard curve (Appendix C). Fluorescence was corrected for inner filter effect [101] due to the error introduced by strong BHQ1 absorbance in the 1:20 and 1:30 samples. Experiment performed at room temperature.

Continuing to expand the investigation into nanobeacons of different peptide-QD ratios, the quenching trend is observed to be consistent with and without Tween, and each nanobeacon can be activated by a large degree, either approaching or exceeding the unquenched QD fluorescence (Fig. 5.11). This data fits well into the working hypothesis as previously explained (Fig. 5.10). At 1:2 ratio, an open peptide conformation confers almost no quenching. The inconsistencies in the activated signals compared to previous experiments and expectations may possibly be attributed to temperature effects (room temperature in current experiment vs. 37°C in previous experiments).
MMP7 Activation

Fig. 5.12 MMP-7 activation of 1:10 nanobeacon. Significant activation of the 1:10 nanobeacon by MMP-7 is observed under optimized cleavage condition with Cremophor. This is in contrast to previous experiments showing very poor activation (Appendix D). Lack of further signal increase upon injection of Proteinase K suggests that complete cleavage is highly likely. Activation did not reach the expected fluorescence level of unquenched QDs (5.7×10⁶) possibly due to fluorescence decrease in MMP buffer or by MMP-7. Assay used MMP buffer, 0.25 μM 1:10 nanobeacon (2.5 μM peptide), 73.5 nM MMP-7 enzyme, 0.5% cremophor, and 30.8 μM Proteinase K. Insets show fluorescence spectra over the time course of activation.
Control Experiments

**Fig. 5.13** QD alone control. 0.258 µM of QD used in 1 mL PBS. Tween arrows denote 1 µL injections. 50 µL (30.8 µM) Proteinase K was injected.

Fig. 5.13 replicates the conditions in a previous experiment (Fig. 5.5) showing that QD fluorescence is neither affected by Tween 80 nor increased by Proteinase K. However, either Proteinase K enzyme or its buffer quenched QD. The same is not observed when experiment is performed in borate buffer (Appendix E). Slight increases and decreases of QD fluorescence may be attributed to photo-brightening [102, 103] and temperature effects [104]. QDs were kept at room temperature between 130 min to 340 min and at 37°C during fluorescence measurements.
**Fig. 5.14** Tween cleavage control. Using commercial OmniMMP substrate, the potential cleavage activity of Tween can be refuted. The first 8 min shows stable OmniMMP fluorescence in PBS. Upon injection of 5 µL (0.5%) of Tween 80, the fluorescence is seen to increase slightly then stabilizes immediately. This increase is insignificant when compared to the amount of increase seen upon cleavage (Appendix F, $2.5 \times 10^6$ after complete cleavage). The minute jump may be indicative of peptide conformation change or Tween fluorescence. 8 µM substrate was used in 1 mL PBS. Data is taken from the first 40 min of a cleavage experiment (Appendix F).

**Fig. 5.15** BHQ1 stability against Tween 80. To ensure that Tween does not reduce nanobeacon quenching by decomposing or modifying BHQ1 absorbance, spectra of 1:30 nanobeacon absorbance was monitored in PBS and in PBS with 0.5% Tween. No change in BHQ1 absorbance (broad peak around 500 nm) was observed.
**Fig. 5.16** Characterization of Proteinase K enzyme velocity in different buffers. Assay used 6 μM of OmniMMP substrate in 1mL buffer with 30.8 μM Proteinase K injection.

To ensure that Proteinase K has sufficient activity in the assay conditions used in the studies, OmniMMP was used as a positive control to monitor cleavage activity of Proteinase K in two different buffers (Fig. 5.16). In both cases, fluorescence increase plateaus immediately after Proteinase K injection, indicating complete cleavage. Manufacturer specified 99.5% quenching efficiency for OmniMMP, translating into 200-fold activation. 67-fold activation was achieved in PBS + Tween, while 105-fold was achieved in MMP buffer. The difference may be attributed to slightly different peptide conformation or environmental effects on the Mca fluorophore. The experiments show that Proteinase K can cleave 6 μM of peptides almost instantaneously at the concentrations and conditions chosen, which are used for other cleavage studies in this chapter. Generally, assay conditions use peptide concentrations in the range of 0.5 μM to 7.5 μM.
5.3 Förster Theoretical Analysis

By using FRET theory as described in Section 2.5, it is possible to calculate the separation distance between QDs and BHQ1s with and without detergent. If the hypothesis that detergents improve cleavage efficiency by opening peptide conformation is correct, then the difference in separation distance between when there is detergent and when there is no detergent should be shorter than the length of the peptide. In other words, it should be possible to explain detergent-induced FRET efficiency change by peptide unraveling and the accompanying positional shift of the quenchers.

Based on Ref [105], the geometry of the peptide bond can be drawn as in Fig. 5.17, where the lengths of the bonds are in Å and the angles are in degrees.

![Fig. 5.17 Peptide bond geometry as solved by crystallography.](image)

By simple trigonometry, each amino acid adds about 3.72 Å in length to a peptide. Therefore, the GPLGLARK peptide can be estimated to be about 30 Å, or 3 nm in length when fully extended.
Table 5.1 Förster analysis of donor-acceptor separation distances and FRET efficiencies

<table>
<thead>
<tr>
<th>Experiment Condition</th>
<th>Separation Distance (Å)</th>
<th>Energy Transfer Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Tween</td>
<td>Tween</td>
</tr>
<tr>
<td>1:10 nanobeacon with Tween</td>
<td>34</td>
<td>64</td>
</tr>
<tr>
<td>1:10 nanobeacon with Cremophor EL</td>
<td>35</td>
<td>56</td>
</tr>
<tr>
<td>1:30 nanobeacon with Tween</td>
<td>39</td>
<td>63</td>
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<tr>
<td>†1:2 nanobeacon with Tween</td>
<td>34</td>
<td>N/A</td>
</tr>
<tr>
<td>*1:10 nanobeacon with Tween</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>1:20 nanobeacon with Tween</td>
<td>35</td>
<td>58</td>
</tr>
<tr>
<td>*1:30 nanobeacon with Tween</td>
<td>40</td>
<td>64</td>
</tr>
</tbody>
</table>

† If assuming separation distance of 64 Å with Tween, transfer efficiency = 0.06

* Repeated experiment with different concentration

Other parameters that have been calculated or measured for this analysis include

- \( R_0 = 37 \text{ Å} \) (compared to 42 Å - 55 Å reported by others [12]. The calculated \( R_0 \) is lower than those reported likely due to the low quantum yield of this particular batch of QD)
- \( Q_{D} = 0.0685 \) (Appendix B)
- \( n_{D} = 1.335 \) (\( n_{D} \) of PBS, effect of Tween on refractive index assumed to be negligible. Literature quotes \( dn/dc = 0.1 - 0.15 / \text{g/mL} \) for surfactants [106]. Typical experiments presented here use concentrations of no more than 0.005 \( \text{g/mL} \))

Table 5.1 shows that the reduced nanobeacon quenching efficiency observed upon the addition of surfactant can be accounted for by a separation distance change that is less than the peptide length, consistent with our hypothesis that Tween reduces quenching by changing peptide conformation.
Estimations and assumptions in the calculations

- $F_D$ and $F_{DA}$ are estimates since QD fluorescence is environment-sensitive and peak height was used. Proteinase K activation of nanobeacon is assumed to be equivalent to $F_D$.

- $\kappa^2$ is assumed following Mattoussi’s analysis [12]. $n$ is estimated.

- $n_D$ may be changed by local concentration effects, this may have a non-trivial effect since it operates to the power of 4 (equation (2-2))

- $J(\lambda)$ and $Q_D$ are estimates since a minor QD emission tail was not included

**Fig. 5.18** Experimental FRET data matches well with theory. QD-peptide separation is between 3 to 4 nm without Tween, and about 6 nm with Tween.

Plotting the data in Table 5.1 against theoretical curves shows that the two are in good agreement (Fig. 5.18), which supports the hypothesis that detergents open peptide conformation. Plotting the theoretical curves also give some insights into key parameters for nanobeacon design. Supposedly, activation becomes more non-linear as $r < R_o$. This is desirable because more quenching is achieved with fewer quenchers, which can translate into better sensitivity. To achieve this, either $r$ can be decreased through peptide design or $R_o$ can be increased by increasing quantum yield and spectral overlap.
5.4 Michaelis-Menten Analysis

This section presents results of cleavage simulations based on the widely accepted Michaelis-Menten model. While the methodology has not been validated, the simulations were highly instrumental and successful in guiding the optimization of cleavage conditions.

The Michaelis-Menten equation is written as

\[ V = V_{\text{max}} \frac{[S]}{K_m + [S]} \]  

(5-1) [12]

where \( V \) is the enzyme cleavage velocity, \( V_{\text{max}} \) is the maximum velocity at a given enzyme concentration, \([S]\) is the concentration of the substrate, and \( K_m \) is the Michaelis-Menten constant equivalent to \([S]\) at which \( V = \frac{1}{2} V_{\text{max}} \). In doing cleavage simulation, \( V \) is re-calculated and applied iteratively as \([S]\) decreases in the course of the experiment (Appendix G). In the limited cases that have been investigated thus far, simulations match well with experiments.

The following set of theoretical predictions has been used as guides when choosing cleavage conditions in our experiments.

1. When \([S] \ll k_m\), the enzyme concentration is the strongest factor influencing the amount of time it takes to reach 100% cleavage (Fig. 5.19)

2. When \([S] \gg k_m\), enzyme to substrate ratio is the strongest factor influencing the amount of time it takes to reach 100% cleavage (Fig. 5.22)

3. Nanobeacon cleavage kinetics is different from regular substrates and can be modeled by treating each QD as a substrate that can be cleaved multiple times (Fig. 5.25)
Case [Substrate] ≪ k_m

![Graph showing Michaelis-Menten cleavage simulation of different enzyme and substrate concentrations and ratios in the case when [S] ≪ k_m](image)

**Fig. 5.19** Michaelis-Menten cleavage simulation of different enzyme and substrate concentrations and ratios in the case when [S] ≪ k_m

Simulation parameters:

\[ k_m = 50 \mu M \text{ (literature quotes } 28 \pm 3 \mu M [100]) \]

\[ k_{cat} = 10 \text{ min}^{-1} \text{ (literature quotes } \sim 2.8 \text{ s}^{-1} = \sim 168 \text{ min}^{-1} [64, 107, 108]) \]

<table>
<thead>
<tr>
<th>Curve</th>
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<td>8.25 x 10^{-12} M</td>
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</tr>
<tr>
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<td>82.5 nM</td>
<td>60:1</td>
<td>4.13 x 10^{-12} M</td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td>10 µM</td>
<td>165 nM</td>
<td>60:1</td>
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<tr>
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<td>5 µM</td>
<td>41.3 nM</td>
<td>120:1</td>
<td>2.07 x 10^{-12} M</td>
<td></td>
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<tr>
<td>Purple</td>
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<td>41.3 nM</td>
<td>240:1</td>
<td>4.13 x 10^{-12} M</td>
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</tbody>
</table>

[S][E] is used to characterize an effective concentration because this term determines the rate of ES complex formation, not the sum of [S] and [E].

In this scenario, we see that enzyme concentration is the only factor influencing time-to-complete cleavage (note this is not the same as cleavage rate, red and blue both reach complete cleavage at the same time, but in blue the absolute cleavage amount is greater). This makes
sense because even though decreasing substrate-to-enzyme ratio decreases the total workload of each enzyme, the situation is offset by the fact that the enzymes work at a slower rate due to a decreased average amount of ES complexes.

![Graph](image)

**Fig. 5.20** Experimental data supporting the theoretical simulation in Fig. 5.19. Experiment performed in 10 mM sodium tetraborate buffer.

Experimental data closely correspond to some of the predicted curves. The general trend agrees with theoretical prediction, with enzyme concentration - not ratio - being the main factor controlling percentage cleavage rate. Note that the curves see a sharper increase compared to prediction then plateaus before complete cleavage. The sharper increase indicates that the $k_{cat}$ used in the simulations was too low, in agreement with literature. The plateau prior to 100% cleavage indicates loss of enzyme activity.
Case \([\text{Substrate}] \sim k_m\)

**Fig. 5.21**  Michaelis-Menten simulation of different enzyme and substrate concentrations and ratios in the case when \([S] \sim k_m\)

Simulation parameters:

\[
\begin{align*}
k_m &= 50 \mu\text{M} \quad (\text{literature quotes } 28 \pm 3 \mu\text{M} [100]) \\
k_{\text{cat}} &= 10 \text{ min}^{-1} \quad (\text{literature quotes } \sim 2.8 \text{ s}^{-1} = \sim 168 \text{ min}^{-1} [64, 107, 108])
\end{align*}
\]

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<th>[E]</th>
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<th>([S][E])</th>
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<td>(8.25 \times 10^{-11} \text{ M})</td>
</tr>
<tr>
<td>Red</td>
<td>50 \mu\text{M}</td>
<td>82.5 nM</td>
<td>600:1</td>
<td>(4.13 \times 10^{-11} \text{ M})</td>
</tr>
<tr>
<td>Green</td>
<td>100 \mu\text{M}</td>
<td>165 nM</td>
<td>600:1</td>
<td>(16.5 \times 10^{-11} \text{ M})</td>
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<tr>
<td>Orange</td>
<td>50 \mu\text{M}</td>
<td>41.3 nM</td>
<td>1200:1</td>
<td>(2.07 \times 10^{-11} \text{ M})</td>
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</table>

In this regime, the relationships become more complicated. Both concentration and ratio play a role in determining the time required for cleavage completion. It is worth noting that as \([S]\) approaches \(k_m\), the cleavage velocity is substantially increased, which allows for a very high substrate-to-enzyme ratio.
**Case** [Substrate] >> $k_m$

**Fig. 5.22** Michaelis-Menten simulation of different enzyme and substrate concentrations and ratios in the case when [S] >> $k_m$

Simulation parameters:

- $k_m = 1$ µM (literature quotes $28 \pm 3$ µM [100])
- $k_{cat} = 10$ min\(^{-1}\) (literature quotes $\sim 2.8$ s\(^{-1}\) = $\sim 168$ min\(^{-1}\) [64, 107, 108])

<table>
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<th>[E]</th>
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<tr>
<td>Red</td>
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<td>82.5 nM</td>
<td>60:1</td>
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<tr>
<td>Green</td>
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<td>165 nM</td>
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<td>$16.5 \times 10^{-12}$ M</td>
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<tr>
<td>Orange</td>
<td>5 µM</td>
<td>41.3 nM</td>
<td>120:1</td>
<td>$2.07 \times 10^{-12}$ M</td>
</tr>
</tbody>
</table>

In this regime, the ratio is the dominant factor in determining time-to-complete cleavage. This makes sense as most enzymes would be in the ES complex. Increasing the enzyme-to-substrate ratio means a greater percentage of substrate is being cleaved at any given time.
This series of simulation was instrumental in solving the problem of cleavage condition optimization. Finding an optimal condition was not trivial because it necessitated finding an appropriate enzyme concentration and ratio, and a buffer that satisfied the following:

- good solubility and activity of enzyme
- good solubility and lack of aggregation of nanobeacons
- stable QD fluorescence over the period of a few hours
- Favourable conformation of peptides on nanobeacons

The simulations provided guidance in choosing an enzyme concentration, and signalled warning signs when the matching of simulation to experimental data yielded an unexpected low $K_{\text{cat}}$. This, together with the unexpected plateauing observed in activation experiments (Fig. 5.20), eventually led to the conclusion that MMP-7 was losing activity in borate buffer. As a result, an optimized condition was achieved in MMP buffer (Fig. 5.23).

![Fig. 5.23](image.png)

**Fig. 5.23** Comparison of MMP-7 activity in tetraborate and MMP buffer. At the same concentrations and ratio, OmniMMP can be completely cleaved within 30 min in MMP buffer. This is in contrast to the results in borate buffer, which shows a loss of enzyme activity in less than 30 min. Completion of cleavage was confirmed by lack of signal increase upon Proteinase K (30.8 µM) addition. HPLC data (not shown) shows absence of intact substrate after overnight incubation with Proteinase K, ruling out the possibility that Proteinase K had no activity.
Finally, experimental result under optimized cleavage condition matched well to Michaelis-Menten cleavage simulation using approximate literature values (Fig. 3.19).

![Graph showing comparison of cleavage experiment with theoretical simulation](image)

**Fig. 5.24** Comparison of cleavage experiment with theoretical simulation using literature kinetics parameters. Both $K_m$ and $K_{cat}$ used in simulation are very similar to literature values. Data and theory match to a high degree except in the first 2 minutes, which is a difficult time point to control due to experimental protocol.

Simulation parameters:

\[ k_m = 50 \ \mu M \text{ (literature quotes } 28 \pm 3 \ \mu M \ [100]) \]

\[ k_{cat} = 150 \text{ min}^{-1} \text{ (literature quotes } \sim 2.8 \text{ s}^{-1} = \sim 168 \text{ min}^{-1} \ [64, 107, 108]) \]
**Theoretical Discussion of Nanobeacon Cleavage**

Due to the complexity introduced by having multiple peptides on a single quantum dot, two theoretical models of cleavage behaviour can be hypothesized. In reality, cleavage perhaps proceeds in both manners where the dominating mechanism depends on the regime of enzyme-to-substrate ratio and concentrations.

**Model 1** - An enzyme binds to a peptide on a QD, and then, due to the close proximity of other peptides on the QD, subsequently cleaves each one until the entire QD is activated. We can model each QD as if it’s a regular substrate that takes n times longer to cleave for a QD with n peptides (i.e. $K_{cat}$ is n times smaller when compared to OmniMMP). Cleavage velocity decreases over time due to depletion of QDs with cleavable peptides.

**Model 2** - On average, the number of peptides on all QDs in solution decrease over time at the same rate. i.e. All the QDs have 10 quencher at time 1, then 9 quenchers at time 2, then 8 quenchers at time 3, and so on. Cleavage velocity remains constant over time until almost all quenchers are cleaved because the chance for an enzyme to find a QD with cleavable peptide does not decrease over time.

A simulated time-course of percentage cleavage for Model 2 is depicted in Fig. 5.25. The simulated cleavage for Model 1 would be the same as that of OmniMMP, but at a slower rate.
Fig. 5.25 Simulation of nanobeacon cleavage as modeled by Model 2 in the case when \([\text{substrate}] \ll k_m\).

Simulation parameters:

\[
k_m = 50 \, \mu\text{M} \text{ (literature quotes } 28 \pm 3 \, \mu\text{M} \text{ [100])}
\]

\[
k_{\text{cat}} = 10 \text{ min}^{-1} \text{ (literature quotes } \sim 2.8 \text{ s}^{-1} = \sim 168 \text{ min}^{-1} \text{ [64, 107, 108])}
\]

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<th>[E]</th>
<th>QD ratio</th>
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<td>0.5 , \mu\text{M}</td>
<td>82.5 , \text{nM}</td>
<td>3:1</td>
<td>6:1</td>
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<td>2.5 , \mu\text{M}</td>
<td>82.5 , \text{nM}</td>
<td>3:1</td>
<td>30:1</td>
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<td>1:20 Nanobeacon</td>
<td>0.25 , \mu\text{M}</td>
<td>5 , \mu\text{M}</td>
<td>82.5 , \text{nM}</td>
<td>3:1</td>
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<td>1:30 Nanobeacon</td>
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<td>82.5 , \text{nM}</td>
<td>3:1</td>
<td>90:1</td>
</tr>
</tbody>
</table>

The cleavage rate is dependent on the formation of the ES complex, which is dependent on [E] and [S]. In the case of the nanobeacon, the chance for an enzyme to find a substrate is the same as the chance for an enzyme to find a QD. Therefore, [QD], and not [S] should be used to calculate cleavage velocity. Compared to regular substrate, this has the interesting consequence that cleavage rate is constant throughout the reaction (except when only 1 peptide is left to be cleaved on each QD), and does not plateau as in the usual case of substrate depletion. Using this line of reasoning, nanobeacon was modeled as a substrate that can be cleaved multiple times.
The analysis result shows that an “optimized” enzyme / substrate ratio and concentration for OmniMMP does not readily translate into the same rate of cleavage in nanobeacons. In fact, nanobeacons with higher quencher-QD ratio take longer to reach complete cleavage than OmniMMP even though their enzyme-substrate ratio is better.

To test which model has more validity under at least one experimental condition, prediction of expected fluorescence as a function of number of quenchers (Fig. 5.26) was first generated based on the Förster equations (Section 2.5) and the quenched and activated fluorescence data from Fig. 5.12. This theoretical approach has been verified in the literature [12]. Then, simulations of cleavage based on the two hypothesized models were performed (Fig. 5.27). Model 1 explained the experimental data better than model 2.

**Fig. 5.26** Theoretical curve of nanobeacon fluorescence as a function of number of quenchers predicted by Förster analysis and experimental data at point 0 and 10 from Fig. 5.12.
Fig. 5.27 Michaelis-Menten simulation of nanobeacon cleavage in terms of fluorescence using model 1 and model 2, overlaid with experimental data.

Simulation parameters:

\[ k_m = 50 \, \mu M \text{ for model 1 and } 120 \, \mu M \text{ for model 2 (50 } \mu M \text{ for OmniMMP, Fig. 5.24)} \]

\[ k_{cat} = 16 \, \text{min}^{-1} \text{ for model 1 and } 150 \, \text{min}^{-1} \text{ for model 2 (150 } \text{min}^{-1} \text{ for OmniMMP, Fig. 5.24)} \]

5.5 Discussion on Nanobeacon Dissociation

Because the electrostatic nature of QD-peptide conjugation only came to light months after this series of experiments, controls were not performed to investigate whether some of the observed FRET changes may have originated from nanobeacon dissociation. However, data from electrostatic binding studies (Chapter 4) and this chapter allows for a reasonable assessment of this possibility. While it is probable that dissociation partially accounts for some of the observations described, the general conclusion that peptide conformation is a key factor in nanobeacon cleavage remains supported.
First, it may be argued that the addition of Tween 80 and Cremophor caused the nanobeacons to dissociate rather than changed the peptide conformation. Since most of the characterizations were done in PBS, which has an ionic strength of about 200 mM consisting of monovalent ions, this argument is plausible according to Fig. 4.9. In addition, Tween is typically used at a concentration ten times higher than that of Brij-35 in experiment Fig. 4.9 (0.5% vs. 0.05%), which perhaps makes dissociation even more likely. However, QD-BHQ1 nanobeacon is shown to be much more stable than QD-Rho nanobeacon; the former does not dissociate in MMP buffer whereas the latter does (Fig. 4.7 vs. Fig. 5.8). In addition, dissociation is usually a fast process that can be observed immediately after reagent mixing (Fig. 4.7, Fig. 4.8, Fig. 4.9), but the fluorescence increase observed after adding Tween is a slow process (Fig. 5.5). Data also shows that the 1:2, 1:10, 1:20 and 1:30 nanobeacons increasingly quench even after adding Tween (Fig. 5.11). This is more consistent with peptide conformation change and less consistent with dissociation. If Tween acted by dissociating QDs and peptides, one would expect complete fluorescence restoration upon its addition regardless of the number of quenchers per QD. Theoretical analysis also supports the peptide conformation model better as the separation distance between QD and BHQ1 with and without detergent roughly corresponds to the length of the peptide (Fig. 5.18). Most importantly, dissociation cannot explain the detergent’s necessity in achieving enzyme cleavage (Fig. 5.6, Fig. 5.12 vs. Appendix D). All in all, while it is possible that detergent addition causes QD-peptide dissociation, it most likely happens to a small degree in conjunction with peptide conformation change, and can be treated as errors in the data.

Another possible criticism is that the fluorescence activation observed upon enzyme injection may be due to QD-peptide dissociation as a result of buffer effects rather than enzymatic cleavage. This argument may be addressed by assessing the components of the
buffers and the corresponding dilution as a result of injection into nanobeacon solutions. Proteinase K is stored in 50 mM Tris-HCl, 5 mM CaCl₂, and 50% glycerol. Typically, 50 μL of Proteinase K is injected into 1 mL of total solution. This dilutes the salt down to 2.5 mM Tris-HCl and 0.25 mM CaCl₂, neither of which causes a concern for dissociation because the nanobeacon has been shown to be stable at much higher salt concentrations (Fig. 5.8). However, it may still be argued that the presence of Tween can amplify the destabilizing effects of salts (e.g. Fig. 5.5), particularly that of Ca²⁺. Even though there is some evidence negating this possibility – specifically, the presence of 0.05% Brij-35 did not allow complete activation of a nanobeacon even at 10.25 mM CaCl₂ (Fig. 5.8) – a small possibility remains that some dissociation may occur along with cleavage during injection of Proteinase K. MMP-7 is stored in 50 mM Tris, 5 mM CaCl₂, 300 mM NaCl, 20 μM ZnCl₂, 0.5% Brij-35, and 30% glycerol. Typically, 3.3 μL of MMP-7 is injected into 1 mL MMP buffer with nanobeacons (Fig. 5.12). Because MMP buffer already contains higher concentrations of CaCl₂ and ZnCl₂, the buffer effect of injecting MMP-7 only causes a 2% and 3% change in salt and Brij-35 concentration, respectively. A total of 0.1% glycerol is also added. Overall, there is no reason to suspect that these minute changes in buffer conditions would cause nanobeacon dissociation.

The assurance that MMP-7 activates nanobeacons by cleavage and not dissociation makes a powerful case for the conclusions of this chapter. Even if we assume, for argument’s sake, that Proteinase K activates QDs by buffer effects and dissociation - which would invalidate the key result of detergent’s necessity in cleavage (Fig. 5.6) – this same result is established by MMP-7 cleavage experiments (Fig. 5.12 vs. Appendix D). Indeed, the concern for nanobeacon dissociation can be addressed and it can be shown that the working hypothesis of this chapter remains reasonably supported.
5.6 Conclusion

BHQ1-GPLGLARK-QD nanobeacon was successfully synthesized by electrostatic self-assembly with conjugate valence of between 2 to 30. The necessity of surfactant for QD-BHQ1 nanobeacon cleavage was demonstrated. The proposed mechanism is that surfactants open the conformation of the peptides, which are normally folded due to hydrophobic residues and the conjugated hydrophobic quenchers, to allow for enzyme binding. The explanation was supported by monitoring a change in FRET efficiency with the addition of detergents. Theoretical analysis showed that the inferred change in QD-BHQ1 distance can be accounted for by the peptide length. Complete activation of the nanobeacon by MMP-7 was achieved in the presence of Cremophor EL.
Chapter 6
QD-Rhodamine Nanobeacon

The QD-Rhodamine nanobeacon was synthesized to validate the conclusions drawn based on the QD-BHQ1 nanobeacon. Because QD fluorescence can be sensitive to environmental factors, a change in fluorescence does not necessarily indicate a FRET efficiency change. Unlike BHQ1, Rhodamine fluoresces and allows for better monitoring of the efficiency of energy transfer. While control experiments certainly promoted confidence in the results and conclusions presented in Chapter 5, being able to monitor the ratiometric change between QD and Rhodamine fluorescence would provide even more definitive evidences of cleavage and further strengthen the foregoing conclusions. Two versions of the nanobeacon were synthesized: one by covalent conjugation; the other by electrostatic self-assembly. Surprisingly, results show that neither peptide conformation nor cleavage was significantly influenced by detergent in this nanobeacon. Furthermore, MMP-7 cleavage was only possible in the self-assembled version.

![Diagram of QD-Rhodamine Nanobeacon](image)

**Fig. 6.1** QD-Rhodamine nanobeacon uses Rhodamine in place of BHQ1 in order to allow for monitoring of FRET efficiency using QD-Rhodamine fluorescence ratio.
6.1 Synthesis Characterizations

Two covalently-linked nanobeacons were synthesized with 1:5 and 1:20 QD:Rho ratios. The ratio 1:5 was chosen because at this ratio, every individual nanobeacon has at least one Rhodamine [97]. The ratio 1:20 was chosen to achieve more quenching. Covalent conjugation of Rhodamine-GPLGLARK to QD was confirmed by fluorescence ratio and chromatographic data. No aggregation was observed.

**Fig. 6.2** QD-Rhodamine fluorescence with (green) and without (blue) covalent conjugation. Conjugation was performed using EDC chemistry. Data was taken in MMP buffer using 450 nm excitation.

By fluorescence, it can be seen that the Rhodamine fluorescence increases and the QD fluorescence decreases after covalent conjugation when compared to a simple mixture (Fig. 6.2). The energy transfer is indicative of successful conjugation. It is known that electrostatic self-assembly is disrupted in MMP buffer (Fig. 4.7), which means that QDs and Rho-peptides are freely dissociated without covalent linkage. The 1:5 nanobeacon is further characterized by
HPLC (Fig. 6.3) to ensure the covalent nature of the QD-Rho linkage, and by microscopy (Fig. 6.4) to ensure that the nanobeacon is aggregate-free.

**Fig. 6.3** Chromatographic characterization of 1:5 QD:Rho nanobeacon. From top to bottom, the chromatograms show Rho-peptide alone, QD alone, QD and Rho-peptide simply mixed, and QD and Rho-peptide conjugated. Absorbance spectra at the bottom, from left to right, show QD and Rho simply mixed, QD and Rho conjugated, QD alone, and Rho-peptide alone.

Chromatography data (Fig. 6.3) shows that the conjugated nanobeacon has a negligible amount of free Rho-peptide (no peak at 56 min in the 560 nm channel) and most of them are conjugated to the QDs. In the unconjugated mixture, interpretation of the data is more
complicated since the self-assembly process can be influenced by both the HPLC mobile phase and the column packing (negatively charged silica beads). During the first 20 min of chromatography, water is used to elute QDs. It can be seen that some Rho-peptides self-assembled onto the QDs (peak at 9.63 in 560 nm channel, also seen in absorbance spectrum), but a substantial amount remains in the column through interaction with the silica beads. At a later time, the peptides elute when the mobile phase is switched to ACN/0.1% TFA (56 min peak in 560 nm channel). Overall, HPLC data shows that covalent conjugation was successful.

Fig. 6.4 Fluorescence imaging of 1:5 QD:Rho nanobeacon. Sample shows no sign of aggregation. Image was taken at 40X under excitation of mercury arc lamp. The field of view shows the edge of a droplet sandwiched between two glass coverslips.

Characterizations of nanobeacons synthesized by electrostatic self-assembly are presented mainly in Chapter 4. A direct comparison of QD-Rho fluorescence between covalently-linked and electrostatically-linked nanobeacons shows very little difference in energy transfer between the two in borate buffer (Fig. 6.5).

Fig. 6.5 QD-Rho fluorescence of 1:20 nanobeacon synthesized by covalent conjugation (blue) and electrostatic self-assembly (red). Excitation was done at 450 nm.
Finally, even though the QD chosen and Rhodamine Red are shown to have reasonable spectral overlap (Fig. 6.6), the overlap can be further improved in the future when optimizing nanobeacon performance.
6.2 Effect of Conjugation Method on Cleavage Efficiency

Traditionally, the advantages and disadvantages of different conjugation methods are assessed based on simplicity of protocol, purity of product, and preservation of functions. The underlying assumption is that the final conjugates, apart from stability issues, would be functionally identical regardless of the conjugation method chosen. For the first time, this section reports a system in which conjugation method can influence the functionality of a conjugate, namely its effect on the cleavage efficiency of nanobeacons.

Rho-GPLGLARK cleavage by MMP-7 and Proteinase K

**Fig. 6.7** Cleavage of Rho-GPLGLARK. Chromatograms show Rho-peptide cleavage by MMP-7 and Proteinase K over night, and negative control without proteases.

It can be shown that GPLGLARK can still be cleaved by both MMP-7 and Proteinase K after Rhodamine modification (Fig. 6.7). MMP-7 achieved near complete cleavage while Proteinase K completely cleaved the peptide. In both cases, the cleavage product no longer interacts with the column resin and elute during the water phase. This is to be expected as the peptide fragment with Rhodamine has no positive charges (Rho-GPLG), which would also imply that Rhodamine can indeed successfully dissociate from the QDs after cleavage.
Proteinase K Cleavage of 1:5 Nanobeacon

Cleavage of the nanobeacon by Proteinase K is evidenced by the increase of QD fluorescence and decrease of Rhodamine fluorescence (Fig. 6.8). Comparing the post-cleavage fluorescence signal to the signal of the unconjugated control suggests near-complete cleavage. The Rhodamine-QD fluorescence ratio decreased from about 1.3 to 0.3. Proteinase K cleavage is slower than observed in other experiments due to components in the MMP buffer (casual observation). Cremophor (0.5%) was added to the sample to see if cleavage would speed up. However, no pronounced effect was observed.

**Fig. 6.8** Proteinase K cleavage of 1:5 QD:Rho nanobeacon. 13.3 nM nanobeacon and 30.8 μM (50 μL) Proteinase K was used in 1 mL MMP buffer. Excitation done at 450 nm. Top figures show full spectra taken at various time points after Proteinase K injection.
Complementing fluorescence data, chromatography data presents even more definitive proof of complete nanobeacon cleavage by Proteinase K (Fig. 6.9). In the intact nanobeacon, Rhodamine elutes at the same time as QDs at 9.5 min. After Proteinase K cleavage, Rhodamine separates from QDs and eluted alone at 27.28 min. The same peak in the 400 nm channel represents noise that originated from the absorption tail of Rhodamine.

**QD-Rhodamine Nanobeacon**  
**Proteinase K-cleaved Nanobeacon**

![HPLC Data](image)

**Fig. 6.9** HPLC data shows complete dissociation of Rhodamines from QDs after cleavage. The assay was carried out in MMP cleavage buffer.
Proteinase K Cleavage of Covalent and Electrostatic 1:20 Nanobeacons

The purpose of this experiment was to begin an investigation into the differences in cleavage kinetics between the covalently conjugated and the electrostatically self-assembled nanobeacons.

**Fig. 6.10** Cleavage kinetics difference between covalently-linked and self-assembled nanobeacons. Top figures show the time-course of QD and Rho fluorescence. Bottom figures show the full spectra taken at each time point. Peaks at 535 nm and 590 originate from QD and Rho, respectively. Cleavage was done using 66.7 nM covalent nanobeacon, 73.4 nM self-assembled nanobeacon, and 30.8 μM Proteinase K in 1mL 10 mM sodium tetraborate buffer. 5 μL of Cremophor EL was injected to reach a final concentration of 0.5%.
The initial observation that motivated this study was the slow cleavage of the covalent 1:5 QD-Rho nanobeacon by Proteinase K (Fig. 6.8) when compared to the self-assembled QD-BHQ1 nanobeacons previously characterized. It was later discovered that the difference in kinetics was largely a result of buffer conditions (Compare Fig. 6.8 to Fig. 6.10). However, as it turns out, even though conjugation method played only a small role in changing the cleavage kinetics of Proteinase K, continued investigation found the surprising result that it, indeed, plays a key role in determining MMP-7 cleavage.

The difference in Proteinase K cleavage kinetics between covalently-conjugated and self-assembled nanobeacons is a small but observable one (Fig. 6.10). While the self-assembled nanobeacon showed almost instant cleavage and immediate stabilization of fluorescence signal, the covalent nanobeacon took 8 min after the initial jump in signal before reaching a plateau. This implies that a small portion of peptides in the covalent nanobeacon are cleaved under different kinetics. PAA can sometimes form multilayer coatings on QDs. Potentially patchy wrapping makes it possible for peptides to access and conjugate to functional groups on one of the deeper coating layers, thus making a portion of the peptides more difficult to cleave. Conversely, in electrostatic self-assembly, the interactions are presumably more flexible, fluid, and longer in range, which would average out the complications introduced by a multilayer coating. This offers one explanation for the small difference observed in cleavage kinetics between nanobeacons employing the two conjugation methods. Addition of Cremophor is observed to slightly increase energy transfer and enhance Rho fluorescence in the self-assembled nanobeacon, presumably due to slight improvement in peptide solubility and absorbance (Appendix A).
MMP-7 Cleavage of Covalent and Electrostatic 1:20 Nanobeacons

A natural step after Proteinase K characterization is to investigate MMP-7 cleavage. This series of experiments show that the electrostatic nanobeacons can be completely cleaved by MMP-7 while the covalent nanobeacons cannot. A buffer injection control was done to show that the FRET decrease observed is not caused by QD-peptide dissociation. While some dissociation was seen, the difference between the cleavage and the control experiment allows for the conclusion that cleavage was definitive.

![Graph showing MMP-7 activity positive control. Cleavage of OmniMMP demonstrates reasonable activity of MMP-7 in the buffer condition chosen. Experiment was done in 60 µL cuvette with 0.5% cremophor, 0.5 mM sodium tetraborate, 33.3 µM (0.1 µL) OmniMMP substrate, and 410 nM (3.3 µL) MMP-7 and 30.8 µM (3 µL) pro-K injections.](image)

Choosing a new cleavage condition than the one previously established for QD-BHQ1 nanobeacons was necessary because QD-Rho nanobeacons are known to dissociate in MMP buffer (Fig. 4.7). Water was chosen to avoid QD-peptide dissociation. Cremophor was added to increase the solubility of the peptide substrate. 0.5 mM of sodium tetraborate was added to
simulate QD nanobeacon injection. To compensate for the expected decrease in enzyme activity due to lack of stabilizing salts ZnCl$_2$ and CaCl$_2$, enzyme concentration was increased. In this condition, MMP-7 was able to nearly cleave all the OmniMMP substrate within 30 min, and adding Proteinase K resulted in little further cleavage (Fig. 6.11).

![Graph showing MMP-7 cleavage of self-assembled 1:20 QD:Rho nanobeacon](image)

**Fig. 6.12** MMP-7 cleavage of self-assembled 1:20 QD:Rho nanobeacon. Assay used 67 nM nanobeacon (3 µL, stored in 10 mM borate buffer), 410 nM (3.3 µL) MMP-7, and 30.8 µM (3 µL) Proteinase K in 60 µL water.

Similarly, under the same cleavage condition, complete activation of the electrostatically self-assembled 1:20 QD-Rho nanobeacon by MMP-7 can be achieved (Fig. 6.12). Adding Proteinase-K quenched the QD and had little effect on Rhodamine fluorescence, indicating no further cleavage.
Fig. 6.13  MMP-7 cleavage of covalently-conjugated 1:20 QD-Rho nanobeacon. Assay used 67 nM nanobeacon (3 µL, stored in 10 mM borate buffer), 410 nM (3.3 µL) MMP-7, and 30.8 µM (3 µL) Proteinase K in 60 µL water.

Fig. 6.14  Negative control of 1:20 QD-Rho nanobeacon cleavage. Assay used 67 nM nanobeacon (3 µL, stored in 10 mM borate buffer), 3 µL MMP buffer injection, and 30.8 µM (3 µL) Proteinase K in 60 µL water.
In contrast to the self-assembled nanobeacon, no MMP-7 cleavage was seen of the covalently-conjugated nanobeacon under identical conditions (Fig. 6.13). QD fluorescence activated only minimally and the Rhodamine fluorescence is seen to follow QD from 10 to 40 min, indicating continued energy transfer. Only upon the injection of Proteinase K did the nanobeacon cleave completely.

Because the MMP-7 enzyme as supplied is stored in a buffer containing glycerol, detergent, ZnCl$_2$ and CaCl$_2$, it is possible that MMP-7 injection simply dissociated the nanobeacon without cleavage (Fig. 6.12). A negative control experiment was done to check for QD-Rho dissociation as a result of buffer injection (Fig. 6.14). Results show that some dissociation did occur due to the small volume of MMP buffer added. However, a much larger change in fluorescence was observed when MMP-7 enzyme was injected (Fig. 6.12), strongly suggesting cleavage by MMP-7. It is unclear why a much more dramatic QD quenching by Proteinase K was seen in the MMP-7 cleavage experiment of the self-assembled nanobeacon compared to the covalent nanobeacon or the control experiment.
6.3 Discussion

Several interesting results were found in the characterization of the QD-Rho nanobeacon in this chapter. Similar to the results found with the QD-BHQ1 nanobeacon, complete cleavage of the QD-Rho nanobeacon by MMP-7 was achieved. However, in contrast to the conclusions drawn with the QD-BHQ1 nanobeacon, detergent played no role in determining the cleavage of the QD-Rho nanobeacon (Fig. 6.8, Fig. 6.10, Fig. 6.12). It was also found that conjugation method can drastically change cleavage, but the effect was only apparent for MMP-7 and not for Proteinase K (Fig. 6.12, Fig. 6.13). Taken in conjunction with the findings in Chapter 5, these results can lead to a mechanistic understanding of nanobeacon cleavage (Table 6.1).

Table 6.1 Enzymatic cleavage of different nanobeacons

<table>
<thead>
<tr>
<th>Acceptor:</th>
<th>Conjugation:</th>
<th>Detergent:</th>
<th>BHQ1</th>
<th>Rhodamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Electrostatic</td>
<td>Yes</td>
<td>No</td>
<td>Electrostatic</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>O</td>
<td>X</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>MMP-7</td>
<td>O</td>
<td>X</td>
<td>O</td>
<td>X</td>
</tr>
</tbody>
</table>


The immediately apparent difference between the QD-BHQ1 and QD-Rho nanobeacons is that the former is affected by detergent while the latter is not. Detergent induces little observable changes in QD-Rho, but influences both FRET efficiency and cleavage efficiency in QD-BHQ1. It may be deduced that the peptides in QD-Rho maintain an open conformation without requiring a detergent. Comparison of hydrophobicity between BHQ1 and Rhodamine supports this notion because Rhodamine was found to be much more hydrophilic (Fig. 6.15) than BHQ1. The hydrophobicity of BHQ1 likely causes it to interact more with the leucine residues on the peptides, thus forming an unfavourable conformation for cleavage. Moreover, this
difference in hydrophobicity can also explain the relative stability of QD-BHQ1 against dissociation when compared to QD-Rho (Fig. 4.7 vs. Fig. 5.8). Because BHQ1-GPGLARK is more hydrophobic than Rho-GPGLARK, presumably there is a higher energy barrier for it to dissociate from the QD surface, thus making the conjugate more stable.

**Fig. 6.15** Hydrophobicity characterization of BHQ1 and Rhodamine. The HPLC gradient is detailed in Section 3.3, Method 2. The longer retention of BHQ1 implies stronger hydrophobicity.

The second finding in QD-Rho is that conjugation method plays an important role in determining cleavage. At first glance, this may seem like an independent finding distinct from the factor of peptide conformation. However, it is possible that the two share the same mechanistic origin. It can be proposed that, ultimately, both factors exert their influences as a consequence of QD steric effects and peptide conformational flexibility.

It was previously suspected that steric hindrance may be a cause for poor nanobeacon cleavage [8, 13, 107]. However, no extensive study has been done to date to investigate this hypothesis. By examining the relevant dimensions and chemistry of the important players in the cleavage process, we can begin to assess how the data presented here might fit into the steric
hindrance explanation. The size of PAA QDs is between 12-13 nm in diameter (unpublished data); the length of GPLGLARK is about 3 nm when fully extended (Section 5.3); the hydrodynamic radius of MMP-7 is between 2.0 – 2.1 nm [109]; and Proteinase K is assumed to be slightly larger than MMP-7 based on molecular weight (28.9 kDa vs. 20.4 kDa for MMP-7). Like many other proteases, MMP-7 has a binding site that is roughly the shape of a “cleft” or a “groove” that fits an extended strand of peptide [110-112]. The binding site is seven amino acids long covering P4-P3’ using the nomenclature of Schechter (Fig. 6.16) [63, 111].

![Peptide and Notation](image)

**Fig. 6.16** Proteolytic nomenclature of amino acid position in a cleavable peptide.

Of these sites, the most well-defined subsite is at the S1’ position [113, 114]. Having a highly hydrophobic residue such as Leu at P1’ is known to be extremely important for cleavage. Other positions, while having an influence on cleavage velocity, do not prevent cleavage even when not properly bound [63]. The further away from the cleavage site, the less effect a position has. Proteinase K, in contrast, has a much less-defined pocket due to its broad substrate specificity, and allows a variety of peptide conformations to bind [115-118].

First, let’s examine how sterics can explain the cleavage difference between covalently-linked and self-assembled QD-Rho nanobeacons. When the peptide is in solution, MMP-7 can freely bind to the peptide for proteolysis (Fig. 6.7). However, when the peptide is attached to a QD, the size of MMP-7 may prevent it from coming close enough to match the amino acids of the peptide to their proper binding subsites. Assuming that the peptide extends perpendicular to the QD surface, which is supported by studies in Chapter 5, the cleavage site would be about 1.5
nm from the QD. However, the S1’ subsite is about 2 nm from the edge of MMP-7, which does not provide enough space for binding. In contrast to covalent linkage, electrostatic binding is longer in range and presumably can be thought of as an elastic bond that allows stretching according to Coulomb’s Law. It is conceivable that MMP-7 can slightly stretch the peptide into position for cleavage. Unlike MMP-7, Proteinase K is more flexible both in terms of binding site and peptide conformation. It is able to cleave GPLGLARK at positions as far as 2.7 nm away from the QD surface (Section 3.1), allowing it to avoid steric hindrance even in the covalent nanobeacon.

It may be argued that the peptide sits parallel to the QD surface and that MMP-7 should be able to cleave the peptide by facing the binding “cleft” to the QD surface. While this peptide configuration is less well supported by data, it is still worth some considerations. The same line of reasoning used previously can be similarly applied. Covalent bond restricts the azimuth angle of the peptide and pins lysine down to the surface of the QD, which can interfere with enzyme binding. In contrast, the flexibility provided by electrostatic binding potentially makes it possible for MMP-7 to shift and rotate the peptide into its binding site.

Finally, some may argue that in the cleavage of self-assembled nanobeacons, the binding sites of MMP-7 simply provided a favourable local environment for the peptides, which in turn promoted their dissociation from the QDs to allow cleavage. This argument can be refuted by the findings of Chapter 5. If cleavage happened through the promotion of dissociation, then detergent would not have been necessary for the cleavage of QD-BHQ1. Extending upon this line of logic, an even more important implication of the experiments in Chapter 5 is that when designing a nanobeacon, the commonly used approach of adding a spacer may not always
address the issue of sterics. As we have seen, even with the flexibility of an electrostatic bond, neither Proteinase K nor MMP-7 can approach the QD close enough to access the peptide when it is raveled up. This means that if the conformation of the peptide, including the spacer, is not in a relatively open form, sterics would continue to inhibit cleavage. In summary, peptide conformation is an important factor to consider in nanobeacon designs.

A hallmark feature of peptides is that they are much smaller than proteins. Therefore, it is not unreasonable to presume that the findings presented here should apply to other nanoparticle-peptide conjugates outside the context of nanobeacons. For example, in applications where peptides are used as epitopes for nanoparticle cell-targeting, sterics may prevent cell receptors from properly binding to the targeting peptides. The results in this work suggest that peptide solubility, conjugation strategy, and detailed biochemistry of the targeting function should be taken into consideration when designing nanoparticle-peptide based probes.

6.4 Conclusion

QD-Rho nanobeacon was successfully synthesized both by covalent conjugation and electrostatic self-assembly. Complete activation by MMP-7 was achieved, supporting the results obtained with the QD-BHQ1 nanobeacon. However, activation was only possible in the self-assembled version, whereas it is inhibited if covalent conjugation were employed. This observation is attributed to the flexibility of the electrostatic bond, which presumably allowed MMP-7 to overcome QD steric hindrance by slightly stretching the bond during enzymatic binding.
Chapter 7
Conclusion

As with any nanoprobe, there is more than meets the eye in the design of a nanobeacon. The promise of enhanced sophistication in functionality made by nanotechnology comes at the cost of increased complexity. This thesis investigated a self-assembly method in synthesizing nanobeacons, demonstrated the functionality of nanobeacons in solution, and examined the factors that influence proteolysis and their underlying mechanisms.

Electrostatic self-assembly was shown to be a highly attractive conjugation strategy for synthesizing nanobeacons. It satisfies many criteria of an ideal conjugation technique, including simplicity, high yield, valence control, function preservation, and stability. Hydrophobic forces were found to play a role in the self-assembly process. Outside of nanobeacons, the conjugation technique is anticipated to be able to greatly facilitate the optimization of future QD-peptide bioconjugates.

Through both QD-BHQ1 and QD-Rho, the basic functionalities of nanobeacons were demonstrated, including synthesis with precise valence control, valence-dependent quenching, and complete signal activation by MMP-7 proteolysis. The 1:10 QD-BHQ1 and 1:20 QD-Rho nanobeacons achieved signal activations of 2.2 fold and 3.6 fold, respectively. Future generations of nanobeacons can be improved by optimizing quenching efficiency.

Finally, the main contribution of this thesis is in uncovering the factors important for nanobeacon proteolysis that have been elusive so far. Specifically, peptide conformation and
conjugation method were found to be key in determining nanobeacon cleavage. Both factors can be linked to a single mechanistic explanation of QD steric hindrance. This insight into the workings of a nanobeacon will help to better translate nanobeacons from concept into functional probes. In the wider context of the field, steric hindrance and bond flexibility may be worthwhile considerations in the design of nanoparticle-peptide bioconjugates in general.

The future direction of this work would consist of both fundamental studies and nanobeacon optimization. More investigation into the differences between covalent conjugation and electrostatic binding is needed to support the notion that the root of the difference is in their bonding flexibility, which influences the functionality of the final probe. For example, insights can be gleaned by studying the donor-acceptor distance and the nanobeacon cleavage efficiency with increasing charges on the peptide, which presumably makes the electrostatic bond more similar in stiffness and strength to a covalent bond. With a more detailed understanding of nanobeacon cleavage elucidated here, next-generation nanobeacons can be better optimized for \textit{in vivo} applications. Specifically, spectral overlap should be maximized to increase quenching, and conjugate valence should be optimized for the trade-off between sensitivity and signal-to-background ratio. Non-linear activation and specificity of the nanobeacon should also be investigated. Overcoming these challenges would eventually set the nanobeacon unto the course to be applied in cells and in \textit{in vivo} models.

Eventually, challenges of the \textit{in vivo} environment will also need to be addressed. Beyond delivery and toxicity issues that all QD-based probes face, the studies presented here imply that the functionality of a nanobeacon can be inhibited by the environment due to peptide conformational change. For example, in blood, salt conditions and the various proteins that may
adsorb to the QD surface are factors to consider, as are the acidic pH and increased interstitial pressure of the tumor microenvironment. Therefore, work in better controlling peptide conformation through intra-molecular forces such as electrostatic or hydrophobic interactions may be inevitable for the proper functioning of nanobeacons in an \textit{in vivo} environment.
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Appendices

Appendix A  Solubility of Rho-GPLGLARK

Solubility of Rho-GPLGLARK can be monitored by absorbance and fluorescence. When the solubility is low, a reduction in both can be observed.

Beyond solubility characterization, the data also brings additional evidence that QD and Rho-peptide self-assembles through electrostatic interaction. Rho-peptide alone in borate buffer is not completely soluble as seen by the low absorbance and fluorescence (self-quenching). Solubility is improved upon the addition of either Cremophor EL or Tween 80, observed as an increase in both absorbance and fluorescence. The same effect can be achieved by adding QDs, supporting the notion that QD and Rho-peptides self-assemble by electrostatics, thereby solubilizing Rho-peptides at the same time. Rho denotes Rho-GPLGLARK in the figures. All samples were prepared at the same Rho-peptide concentration (0.5 nmol in 80 µL 10 mM tetraborate buffer). 0.5% Tween and Cremophor were used.
Appendix B  QD Quantum Yield Measurement

QD Quantum yield measured in PBS for theoretical FRET analysis. Fluorescein has a quantum yield of 0.79 in ethanol using 425 nm excitation. Quantum yield was measured using the following equation,

$$\Phi_X = \Phi_{ST} \left( \frac{Grad_X}{Grad_{ST}} \right) \left( \frac{\eta_X^2}{\eta_{ST}^2} \right)$$

where $\Phi$ is quantum yield, the subscripts X and ST denote the test sample and standard, respectively, Grad is gradient, equivalent to the slope of the integrated photoluminescence vs. absorption, and $\eta$ is index of refraction of the solvents.
Appendix C  QD Fluorescence Standard Curve

Standard curve for QD fluorescence in PBS buffer. This curve is used to calculate the expected nanobeacon fluorescence after complete cleavage. It was found that readings above $10^7$ is not linear, therefore all fluorescence measurements were kept below this limit.
Appendix D  Failed nanobeacon cleavage conditions

This appendix documents all the failed cleavage conditions that have been tested for QD-BHQ1 nanobeacon activation. These experiments serve as contrast to the successful cleavage experiment presented in Chapter 5 (Fig. 5.12), and highlight the importance of Cremophor in nanobeacon activation. No detergent was used in any of the assays in this section.

1:10 nanobeacon activation in MMP buffer (overnight)

![Graph showing failed cleavage conditions for 1:10 ratio with and without MMP7](image1)

1:5 nanobeacon activation in MMP buffer (overnight)

![Graph showing failed cleavage conditions for 1:5 ratio with and without MMP7](image2)
1:2 nanobeacon activation in MMP buffer (overnight)

With MMP7

Without MMP7

1:2 nanobeacon activation in KB medium

Cell-incubated medium (MMP7)

Fresh medium (no MMP7)
Nanobeacon activation in KB medium with 5% trypsin

1:5 Nanobeacon

1:10 Nanobeacon

1:2 Nanobeacon

1:5 nanobeacon activation MMP buffer with MMP-7

18Hr

38Hrs

18Hr
Appendix E  Proteinase K quenching of QD

In this experiment, MMP-7 cleavage of 1:10 QD-BHQ1 nanobeacon was attempted. However, it was later found that borate buffer was not optimal for MMP-7 enzyme. Fluorescence increased only by 1.8 fold after 130 min of assay time. An interesting observation in this experiment is that Proteinase K quenching of QD is averted in borate buffer. Substrate-enzyme ratio was 36:1. Assay used 0.25 µM nanobeacon (2.5 µM cleavable peptides), and 73.5 nM MMP-7.
Appendix F  Tween does not cleave peptide

First 40 min of this data is presented in Fig. 5.14. Assay used 8 µM of OmniMMP substrate in 1 mL PBS. 5 µL of Tween was injected at 8 min. 24.5 pmol of MMP-7 was injected at 40 min (resulting in 24.5 nM concentration) and again (another 24.5 nM) at 250 min. Finally Proteinase K (30.8 µM) was injected at 380 min to find the activation upper bound. 105-fold activation was achieved in total. Results suggest that Tween does not cleave the peptide substrate, as expected.
Appendix G  Sample Michaelis-Menten simulation

This appendix presents an example of the Michaelis-Menten simulation done in Section 5.4 using Microsoft Excel. Both formulas and the simulation result are presented. [S], V, and [P] denote substrate concentration, enzyme velocity, and cleavage product concentration, respectively. K_m, V_max, and initial [S] were chosen arbitrarily.

Excel Formula

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