

Optimized linkage and quenching strategies for quantum dot molecular beacons

Nathaniel C. Cady*, Aaron D. Strickland, Carl A. Batt

Department of Food Science, Cornell University, 317 Stocking Hall, Ithaca, NY 14850, USA

Received 5 July 2006; accepted 7 September 2006

Available online 17 September 2006

Abstract

Quantum dot (QD) molecular beacons were explored for sequence-specific DNA detection. The effectiveness of multiple linkage strategies and fluorescence quenchers were compared in hybridization-based assays. To compare linkage strategies, covalent amide linkage and streptavidin–biotin binding were used to link semiconductor QDs to molecular beacon DNA. Amide-linked beacons showed a 57% greater fluorescence increase than streptavidin-linked beacons when hybridized to 200 pmol of target DNA. The specificity of the molecular beacons, however, was similar for both linkage methods. Hybridization of both QD molecular beacons with non-complementary target DNA resulted in approximately 50% lower fluorescence intensity than hybridization with complementary DNA. The effectiveness of different quencher moieties was also evaluated. Iowa Black and 1.4 nm Nanogold-quenched molecular beacons exhibited approximately 2-fold greater fluorescence increases than dabcyI-quenched beacons when hybridized to complementary target. Specificity for target DNA was also confirmed through hybridization assays with non-complementary DNA. To provide insight into differences between the QD molecular beacons and the linkage strategies used, the hydrodynamic radius of each was measured. These measurements indicated that the larger radius of the streptavidin QDs (13.5 nm) than the carboxyl QDs (7 nm) could have a negative effect on FRET-based quenching for QD molecular beacons. These data outline the importance of choosing proper linkage methods and quencher moieties for creating high-quality QD molecular beacons.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Quantum dots; Biosensor; Molecular beacon; DNA; Detection

1. Introduction

Semiconductor quantum dots (QDs) have become attractive fluorophores for biosensing strategies. QDs are much brighter and more photostable than organic fluorophores, have narrow emission spectra (25–30 nm full-width at half-maximum), and have broad absorption spectra [1–3]. Because of the broad absorption spectra and large emission shift, QDs can be excited at wavelengths far-removed from their emission peak. Additionally, nearly all QDs of different emission peaks can be excited using a single, short-wavelength excitation source. There have been many recent reports of using QDs for biosensing applications [1,2,4,5], especially for multiplex detection strategies [3,5–7].

The sensitivity of target detection is a function of the intensity of the signal and the background noise of the system. One method of increasing signal-to-noise ratios involves the use of fluorescence resonance energy transfer (FRET). FRET is a distance-dependent phenomenon that occurs when a donor fluorophore and an acceptor chromophore (quencher) are in close proximity [8]. When the fluorophore and quencher are within a certain distance from each other, excitation energy can be transferred from the fluorophore to the quencher, preventing fluorescence emission. The distance at which there is a 50% transfer in energy is known as the Förster Radius (R_o):

$$R_o = 9.78 \times 10^3 [k^2 n^2 Q_o J(\lambda)]^{1/6}.$$

R_o is dependent upon (k) the orientation factor for dipole–dipole interactions, (n) the refractive index of the medium, (Q_o) the quantum efficiency of the donor, and ($J(\lambda)$) the spectral overlap integral between the donor and

*Corresponding author. Tel.: +1 518 437 8686; fax: +1 518 437 8603.
E-mail address: ncady@uamail.albany.edu (N.C. Cady).

acceptor [4,9]. Once R_0 is known, the FRET efficiency (E) can be calculated for various distances (R) between the donor and the acceptor [9]:

$$E = \frac{R_0^6}{R_0^6 + R^6}.$$

Multiple detection techniques have been devised to harness this effect, including molecular beacons [10–13]. Molecular beacons are comprised of a fluorophore and a quencher moiety attached to opposite ends of a single stranded DNA oligonucleotide. The sequence of the oligonucleotide is designed such that it preferentially base pairs with itself, forming a stem-loop structure. Included inside the loop region is a probe sequence that can bind to a complementary target DNA sequence. This hybridization event causes the stem-loop structure to open and spatially distance the fluorophore from the quencher. This decreases the FRET efficiency and results in increased emission from the fluorophore (donor). Molecular beacons are extremely target-specific, primarily because of the competition between internal hybridization within the stem structure and hybridization between the target and the loop structure [11]. These dynamic interactions require specific hybridization between the target and loop structure to stabilize the molecular beacon in the open position. Additionally the stem and loop length of the molecular beacon can be altered to achieve either higher specificity and/or increased kinetic rate constants [13]. These features make molecular beacons attractive for biosensor detection applications.

To this end, we have developed several QD-based molecular beacons to be used for DNA-based detection strategies. Although the use of QDs as fluorescence donors in molecular beacons is not unprecedented [9], we present here a variety of methods for QD-molecular beacon synthesis, as well as a comparison of different fluorescent quenchers. Additionally, we demonstrate the effective use of QD-molecular beacons for DNA sequence detection.

2. Materials and methods

2.1. Reagents

Phosphate buffered saline (PBS) pH 7.4 was obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was obtained from New England Biolabs (Beverly, MA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Pierce Biotechnology Inc. (Rockford, IL). Carboxyl-modified and streptavidin-modified 525 nm quantum dots were obtained from Quantum Dot Corporation (Hayward, CA). All DNA (oligonucleotide primers and molecular beacon oligonucleotides) were obtained from Integrated DNA Technologies (Coralville, IA). Mono-sulfo-NHS Nanogold conjugates (1.4 nm) were obtained from Nanoprobes Inc. (Yaphank, NY). Microcon molecular weight cut-off (MWCO) spin filters were obtained from Millipore

Corporation (Bedford, MA). PicoGreen fluorescent dye was purchased from Molecular Probes (Eugene, OR).

2.2. Molecular beacon design

The molecular beacon used in this study was designed using the nucleic acid manipulation software Vector NTI (Invitrogen, Carlsbad, CA). The molecular beacon was designed to hybridize to a 17 base portion of the *Salmonella typhimurium* invasin gene (*invA*). Using Vector NTI and BLAST (www.ncbi.nlm.nih.gov/BLAST/) [14], a sequence was selected that did not have significant similarity to other reported sequences and had no predicted secondary structure formation. A 5 base “stem” was added to the 5′ and 3′ ends of this sequence that would enable stem-loop formation through internal hybridization to create the molecular beacon. The resulting sequence is 5′ **CGCTCGTGT**TTATGGGGTCGTT**GAGCG** 3′ where the bold, underlined portions represent the 5 base stem. To determine the effectiveness of this molecular beacon for sequence-specific detection, two DNA sequences were chosen. The first, “invAF2” (5′TGCTGCTTTCTCTACTTAACAGTGC 3′) is complementary to the 5′ end of the *invA* gene, but does not overlap with the molecular beacon sequence. The second, “MB Comp” (5′AACGACCCCATAAACAC3′) is exactly complementary to the loop portion of the molecular beacon.

Molecular beacons were designed to use a quantum dot (QD) as the fluorophore and a variety of quenching moieties to provide fluorescence quenching. These included the Iowa Black FQ quencher (Integrated DNA Technologies), dabcyI, and 1.4 nm gold particles (Nanoprobes). For dabcyI and Iowa Black FQ quenched carboxyl-modified QDs the molecular beacon DNA was modified with a 5′ C6 amino modifier and either a 3′ dabcyI or Iowa Black FQ molecule. For Iowa Black FQ quenched streptavidin-modified QDs the molecular beacon DNA was modified with a 5′ biotin and a 3′ Iowa Black FQ. Finally, for Nanogold quenched molecular beacons the molecular beacon DNA was modified with a 3′ C6 amino modifier (for linkage to the Nanogold) and a 5′ biotin for linkage to streptavidin-modified QDs.

2.3. Quantum dot—molecular beacon synthesis

Several methods were used to attach quantum dots to the molecular beacon DNA backbone. As described above, multiple molecular beacon DNA molecules were synthesized with different attachment chemistries and quencher molecules attached to their 5′ and 3′ ends. The different molecular beacons that were synthesized included dabcyI- and Iowa Black FQ-quenched carboxyl-modified QDs, Iowa Black FQ-quenched streptavidin QDs, and finally Nanogold-quenched streptavidin-modified QDs (as shown in Fig. 1 and listed in Table 2). These represent a range of chemistries available for DNA–QD attachment, and include the most widely used quenchers for molecular

beacons and other FRET-based assays. A list of the DNA molecules used for QD molecular beacon synthesis is provided in Table 1, while the various molecular beacons that were synthesized are shown in Table 2.

For the first set of molecular beacons, Qdot ITK carboxyl-modified 525 nm quantum dots (Quantum Dot Corporation) were covalently linked to the 5' amino linker of molecular beacon DNA modified with both dabcy and Iowa Black FQ. The carboxyl surface groups of the QDs were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) [15] and allowed to react with the 5' amino groups on the DNA. Approximately 100 pmol of Qdots were mixed with 300 nmol of EDC and 2 nmol of amino-labeled DNA in 400 μ L of PBS in a 1 mL glass vial. The mixture was allowed to react for 2 h at room temperature. Unreacted DNA and free EDC were removed from the QD-modified DNA by spin

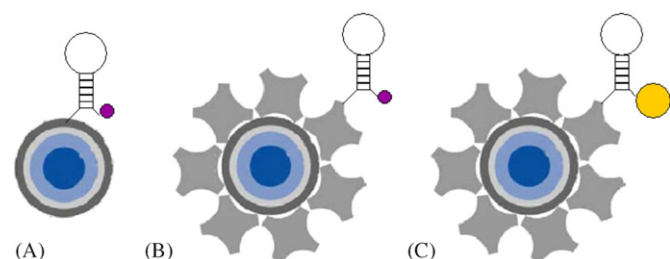


Fig. 1. A representation of the three different quantum dot molecular beacon labeling strategies is shown. Carboxyl-modified quantum dots were modified with amino-labeled molecular beacons (A) while streptavidin-modified quantum dots were modified with biotin-labeled molecular beacons (B). Both dabcy and Iowa Black FQ quenchers (small dark circles) were used, as well as 1.4 nm Nanogold (light colored circle) (C).

filtration in Millipore Microcon 50,000 molecular weight cut-off (MWCO) spin filters. Reactions were spun at $7000 \times g$ for 5 min and the retentate (QD-modified DNA) resuspended in 400 μ L PBS while the flow-through was discarded. This was repeated two times and the final retentate was resuspended in 200 μ L of PBS.

For the second set of molecular beacons, biotinylated molecular beacon DNA was attached to Qdot 525 nm streptavidin conjugate (Quantum Dot Corporation) by the well-established streptavidin–biotin interaction [15]. Approximately 100 pmol of streptavidin-modified QDs were mixed with 1 nmol of biotinylated molecular beacon DNA and 40 μ L of 10 mg/mL BSA in 400 μ L of PBS. The mixture was incubated for 1 h, washed, and resuspended as described above for the carboxyl-modified QD-molecular beacons.

Finally, for preparation of Nanogold-quenched molecular beacons, mono-sulfo-NHS 1.4 nm Nanogold (Nanoprobe Inc.) was modified with amino-labeled molecular beacon DNA. Approximately 1 nmol of mono-sulfo-NHS Nanogold was mixed with 10 nmol of amino-labeled, biotinylated molecular beacon DNA in 300 μ L of PBS. The mixture was incubated for 2 h at room temperature and then quenched with 10 μ L of 10 mM glycine to deactivate any remaining NHS on the surface of the Nanogold. The reaction was then washed two times with PBS in a Millipore Microcon 10,000 MWCO spin filter with centrifugation at $7000 \times g$ and was resuspended in 200 μ L PBS. One hundred micro liters of the resuspended nanogold was mixed with 10 pmol of Qdot 525 nm streptavidin conjugate, 40 μ L of 10 mg/mL BSA, and 400 μ L PBS. This mixture was incubated 1 h at room

Table 1
Comparison of DNA sequences for molecular beacons, target DNA, and PCR primers used

Sequence Name	Sequence (5'–3')
invAF2	5' TGCTGCTTTCTCTACTTAAACAGTGC
invAF2–NH2 labeled	5' NH2–TGCTGCTTTCTCTACTTAAACAGTGC
invAR4	5' CGGCATCGGCTTCAATCAAGAT
Mol Beacon–NH2, Dabcy	5' NH2– <u>CGCTCGT</u> GTTTATGGGGTCGTT <u>GAGCG</u> –Dabcy
Mol Beacon–NH2, Iowa Black FQ	5' NH2– <u>CGCTCGT</u> GTTTATGGGGTCGTT <u>GAGCG</u> –Iowa Black FQ
Mol Beacon–Biotin, Iowa Black FQ	5' Biotin– <u>CGCTCGT</u> GTTTATGGGGTCGTT <u>GAGCG</u> –Iowa Black FQ
Mol Beacon–NH2, Biotin	5' Biotin– <u>CGCTCGT</u> GTTTATGGGGTCGTT <u>GAGCG</u> –NH2
MB Comp	5' AACGACCCCATAAACAC

The tail sequences of the molecular beacons are shown underlined and in bold text. For each molecular beacon DNA backbone used, the 5' and 3' modifications are shown. The following abbreviations are used in the table: NH2—amine group, FAM—fluorescein dye, MB comp—complementary sequence to the loop region of the molecular beacon.

Table 2
QD molecular beacon summary

Molecular beacon name	Fluorophore/quencher	Molecular beacon DNA sequence
Carb-IB	Carboxyl 525 nm Qdot/Iowa Black	5' CGCTCGTGTGTTTATGGGGTCGTTGAGCG
Carb-Dabcy	Carboxyl 525 nm Qdot/Dabcy	5' CGCTCGTGTGTTTATGGGGTCGTTGAGCG
SA-IB	Streptavidin 525 nm Qdot/Iowa Black	5' CGCTCGTGTGTTTATGGGGTCGTTGAGCG
SA-Gold	Streptavidin 525 nm Qdot/1.4 nm Nanogold	5' CGCTCGTGTGTTTATGGGGTCGTTGAGCG

The abbreviated names and compositions of the various molecular beacons are shown.

temperature and was washed two times with PBS in a Millipore Microcon 100,000 MWCO spin filter at $7000 \times g$. The final retentate was resuspended in 200 μ L PBS. All QD-molecular beacons were stored in the dark at 4 °C and were used within 1 month of their initial synthesis.

2.4. Fluorescence measurements and particle sizing

To measure the fluorescence intensity of QD-molecular beacons we used a standard microplate fluorometer (Tecan Genosys FL, Durham, NC). Microplate fluorescence measurements of QD-molecular beacons were made with excitation at 360 nm and detection at 520 nm. Particle sizing was achieved using a Malvern Instruments (Worcestershire, UK) Zetasizer Nano ZS instrument. This instrument uses light scattering to measure the average hydrodynamic radius of particles in solution. Samples were placed in 0.5 mL plastic cuvettes and three measurements consisting of 10 runs with 5 s duration were performed at 25 °C. The instrument was standardized with 1 μ m polystyrene beads and particle size was reported as the average of the three measurements with an error measurement of one standard deviation.

2.5. Calculation of FRET efficiency

The FRET efficiency for 525 nm Qdots with both dabcyI and Iowa Black FQ were calculated for a range of distances. This was done by first calculating the Förster Radius (R_o) as described by the above equation using previously described values reported by Clapp et al. [9,17]. A value of 2/3 was used for the orientation factor for dipole–dipole interactions (k) while a value of 1.40 was used for the refractive index of the medium (n). A value of 0.60 was used for the quantum efficiency of the donor (Q_o) as specified by Quantum Dot Corporation. The spectral overlap integral between the donor and acceptor ($J(\lambda)$), was found by using the Qdot emission spectrum (from Quantum Dot Corporation) and from empirically determined absorbance spectra of dabcyI and Iowa Black FQ. The overlap integral was calculated numerically using a 1 nm step size. The overlap integral for the 525 nm Qdot–Iowa Black FQ interaction was calculated to be 2.62×10^{-13} , and for the 525 nm Qdot–dabcyI interaction, 7.4×10^{-14} . The Förster Radius (R_o) for 525 nm Qdots–Iowa Black FQ was calculated to be 50.2 Å and for 525 nm Qdots–dabcyI, 40.6 Å. Once R_o was calculated, the FRET efficiency (E) was calculated for a variety of distances from 1 to 20 nm using the previously described equation (above).

3. Results

The goal of this research was to develop QD molecular beacons that could be used for DNA detection assays. Although Kim and co-workers have reported the use of QDs for molecular beacons [9], we sought to evaluate different attachment chemistries as well as different

quencher moieties to determine which combination would yield the best molecular sensor. Our initial approach to creating QD molecular beacons was to attach amino-labeled molecular beacon DNA to carboxyl-modified QDs. The molecular beacon DNA sequence was designed to hybridize with a 17 bp portion of the *Salmonella typhimurium invA* gene. One end of the DNA was labeled with Iowa Black FQ for FRET-based quenching of the QD during hairpin formation. Using the zero-length crosslinker, EDC, we were able to successfully attach Iowa Black FQ-labeled molecular beacon DNA to QDs and could visually observe quenching of QD fluorescence (Fig. 2).

Different linkage strategies and compositions for QD molecular beacons were also explored. We used a popular alternative to covalent chemical attachment, linking the QD and DNA by streptavidin–biotin binding. We also used the alternative organic quenchers Iowa Black™ (Integrated DNA Technologies, Coralville, IA) and dabcyI. Since FRET-based quenching can also be achieved through interactions between fluorophores and gold surfaces [18] or gold nanoparticles [19,20], we also developed a QD molecular beacon using 1.4 nm Nanogold as a quencher. The various QD–DNA linkage and molecular beacon quenching strategies that were used are shown schematically in Fig. 1. The abbreviated names and a short description of each QD molecular beacon are shown in Table 2.

To confirm modification of QDs with molecular beacon DNA, we measured the average diameter of the QD molecular beacons and compared them to unmodified QDs and QD conjugates using a laser-based particle-sizing instrument (Malvern Zetasizer Nano ZS) that has

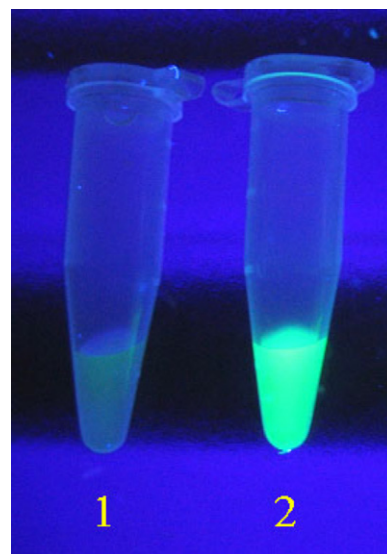


Fig. 2. Fluorescence quenching of quantum dots through the attachment of Iowa Black-modified molecular beacon DNA is shown. Carb-IB quantum dot molecular beacons are shown (left, Tube #1) as well as unmodified Qdot 525 nm ITK quantum dots (right, Tube #2). Images were acquired with a Canon PowerShot A60 digital camera using a Labnet DyNA Light transilluminator as an excitation source. Abbreviated names are described in detail in Table 1.

previously been used to measure nanometer sized particles [21,22]. Because the measured particles are in aqueous solution, these measurements represent the average hydrodynamic diameter of the particles. Light scattering measurements demonstrated an increase in hydrodynamic radius after modification of QDs with molecular beacon DNA. Unmodified carboxyl-QDs had an average hydrodynamic radius of 7 ± 2.2 nm and increased to between 13.5 ± 3.2 and 18 ± 6 nm after modification with various DNA molecules. As expected, these measurements showed that Qdot streptavidin conjugate was significantly larger (15.7 ± 3.7 nm) than the Qdot ITK carboxyl QDs (7 ± 2.2 nm). These measurements agree with the manufacturer's specifications (Quantum Dot Corporation) that Qdot ITK carboxyl QDs are approximately 5 nm in diameter and the streptavidin conjugate is between 10 and 15 nm in diameter. The larger diameter of the streptavidin conjugate is due to the attachment of large, approximately 4 nm diameter [23] streptavidin proteins to the QD surface. Although we could not resolve differences in diameter between DNA-modified and unmodified Qdot streptavidin conjugates, there was a 6-fold increase in hydrodynamic radius between DNA modified (73 ± 16 nm) and unmodified (11 ± 2.4 nm) SA-Gold beacons. This increase could be due to aggregation of particles in solution, or to cross-linking of particles during attachment of the Nanogold-DNA to the Qdot streptavidin conjugate.

To quantitatively compare the effectiveness of the various QD molecular beacons for biosensing, we measured the fluorescence intensity of molecular beacons mixed with complementary and non-complementary DNA. Fixed concentrations of each of the different QD

molecular beacons (approximately 2 pmol for each) were mixed with 200 pmol of complement DNA (MB comp) or non-complement DNA (invAF2). The fluorescence intensity of each mixture was measured and then normalized to the fluorescence intensity of QD molecular beacons without added DNA. The results of this experiment are shown in Fig. 3. The results of the DNA hybridization-based unquenching of QD molecular beacons demonstrate that there are clear differences between the attachment methods used to link the molecular beacon DNA to the QDs, as well as differences in the effectiveness of different quencher moieties. When the same quencher (Iowa Black FQ) was used with different attachment strategies (covalent amino-carboxy versus streptavidin–biotin) a notable difference was seen in hybridization-based unquenching. The covalently linked molecular beacons yielded a 3.3-fold increase in fluorescence while the streptavidin–biotin linked beacons yielded only a 2.1-fold increase in fluorescence. When comparing different quencher moieties, it was clear that the Iowa Black FQ quencher was a better quencher than dabcyl. We used covalent amide linkage for both Iowa Black FQ and dabcyl-labeled molecular beacons. The Iowa Black FQ labeled beacon, however, yielded a 3.3-fold increase in fluorescence (after hybridization with target DNA) whereas the dabcyl labeled beacon yielded only a 1.1-fold increase in fluorescence. This difference could be due to multiple effects including differences in the ability of dabcyl and Iowa Black FQ to effectively quench QDs. In another comparison SA-IB beacons (2.1-fold increase) demonstrated a similar amount of activity to SA-Gold beacons (1.9-fold increase). This demonstrates that Nanogold may have similar quenching properties to Iowa Black FQ in this application.

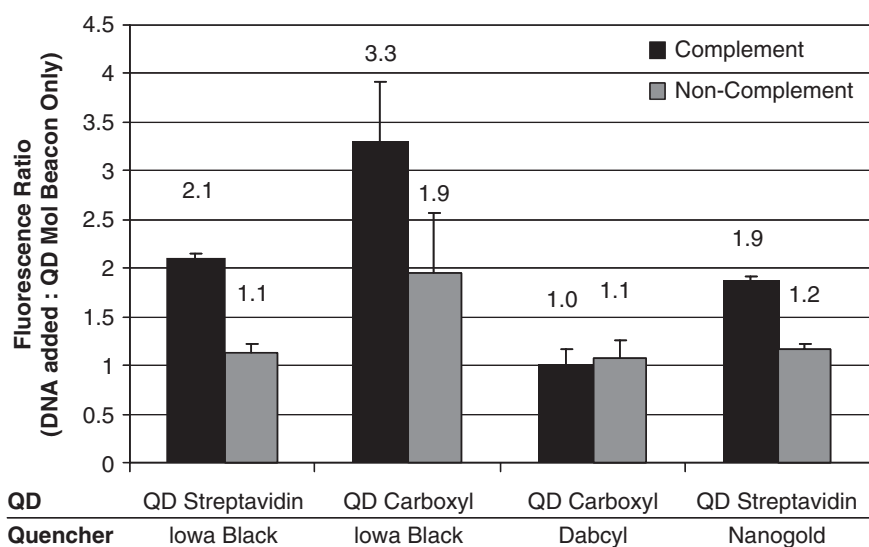


Fig. 3. DNA hybridization-based unquenching of QD molecular beacons. Approximately 2 pmol of QD molecular beacons were mixed with either 200 pmol of complement DNA (MB comp) or 200 pmol of non-complement DNA (invAF2). For each measurement, the fluorescence intensity of the mixture was normalized to the fluorescence intensity of 2 pmol of QD molecular beacons in PBS buffer only. These values are expressed as fluorescence ratios (QD MB with target DNA: QD MB with buffer only). These data represent the average of three separate measurements for each QD molecular beacon and error bars represent one standard deviation.

To better understand if fluorescence unquenching of QD molecular beacons was due to DNA hybridization, we performed additional particle sizing measurements. Both carboxyl-QDs and streptavidin-QDs linked to Iowa Black FQ molecular beacon DNA were mixed with 200 pmol of complement DNA (MB comp) and their average particle size was measured with the Malvern Zetasizer Nano ZS instrument. Molecular beacon modified and unmodified QDs were also measured for comparison (Fig. 4). These results demonstrate that the average hydrodynamic radius of the QD molecular beacons increased upon addition of complementary DNA. The increase in diameter is likely due to hybridization of the molecular beacon DNA with the complement DNA, resulting in an extended DNA structure. For Carb-IB beacons, the average hydrodynamic radius was measured at 13.5 ± 2.8 nm and increased to 15.7 ± 3.8 nm upon addition of complement DNA. For SA-IB beacons the hydrodynamic radius increased from an average of 15.6 ± 3.7 nm to 21 ± 3.1 nm upon addition of complement DNA. This represents a 2.2 nm increase in hydrodynamic radius for the carboxyl QD molecular beacons and a 5.4 nm increase for streptavidin QD molecular beacons. The difference in measured diameter for the Carb-IB beacons, however, is within the standard error, and therefore may not be significant. Taken with the large increase in diameter for the streptavidin QD molecular beacons, however, these data could suggest that fluorescence unquenching is caused by hybridization of complement DNA that opens the beacon hairpin and spatially separates the QD from the quencher.

4. Discussion

This research effort demonstrates that molecular beacons can be synthesized using QDs as a fluorophore and

both organic and inorganic moieties as quenchers. Moreover, these results compare the relative effectiveness of two different binding chemistries for QD molecular beacon synthesis. For this research effort, we used two different methods to attach molecular beacon DNA to semiconductor QDs: (1) covalent linkage of amino-labeled DNA to carboxylated QDs (carboxyl-linked) and (2) linkage of biotinylated DNA to streptavidin-modified QDs (streptavidin-linked). Both of these methods have been demonstrated previously for attachment of DNA to QDs, but the two methods have not been quantitatively compared within a single study. We observed that the linkage method used had measurable effects on the properties of the molecular beacons. As shown in Fig. 4, DNA hybridization-based unquenching of QD molecular beacons yielded larger increases in fluorescence for carboxyl-linked beacons (3.3-fold) than for streptavidin-linked beacons (2.1-fold) when using the same quencher (Iowa Black FQ). This fluorescence increase was mediated by binding of the molecular beacon DNA to single-stranded DNA that was perfectly complementary to the loop region. The difference between the carboxyl-linked and streptavidin-linked molecular beacons could be due to several reasons. First, there could be differences in the number of molecular beacon DNA hairpins attached to the two types of QDs. This could be due to differences in binding efficiency between the two linkage methods and the number of accessible reactive groups (carboxyl or streptavidin) on the QDs. Close packing of molecular beacon DNA on the surface of the QDs could interfere with secondary structure hairpin formation. This could result in inefficient quenching of the QD molecular beacon (in the absence of target) and result in a smaller change in fluorescence intensity during hybridization to target DNA.

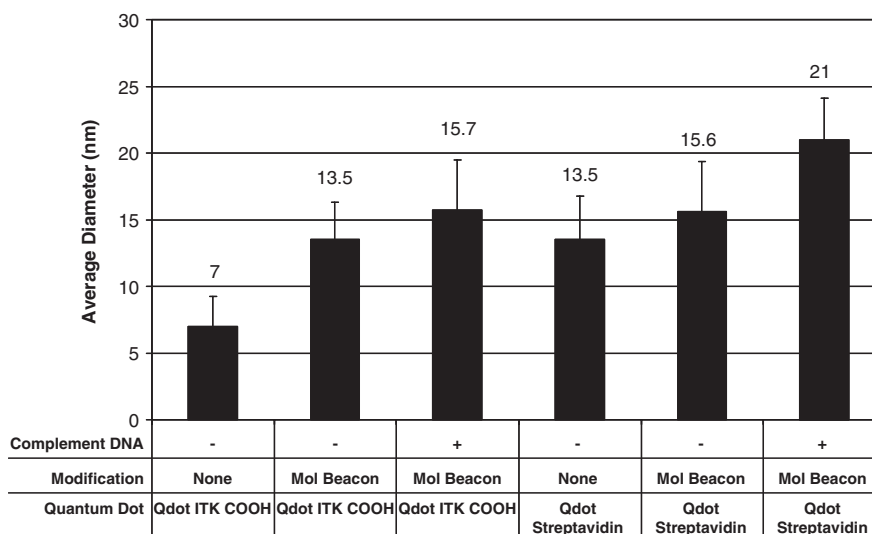


Fig. 4. Particle sizing measurements of QD molecular beacons both alone and hybridized to complement DNA. Approximately 2 pmol of each QD or QD molecular beacon was measured in PBS buffer in a Malvern Instruments Zetasizer (Worcestershire, UK). To some samples, 200 pmol of complement DNA (MB comp) was added to 2 pmol of both Carb-IB and SA-IB beacons to measure changes in hydrodynamic radius due to DNA hybridization. Each measurement is the average of three separate measurements and the error bars represent one standard deviation.

Another difference between the two linkage strategies is the relative distance of the molecular beacon DNA (and therefore the quencher) from the core of the QD. Carboxyl-modified QDs were measured to have an average diameter of 7 nm while streptavidin-modified QDs had an average diameter of 13.5 nm. The difference in diameter is due to the large streptavidin protein molecules that are bound to the surface of the streptavidin-modified QDs. Using large proteins as spacers on the surface of the QD greatly increases the distance between the core of the QD and the quencher molecule, a parameter that is critical to FRET-based quenching. The Förster Radii (R_0) for the QD–Iowa Black FQ pair and the QD–dabcyl pair were calculated to be 5.0 and 4.1 nm, respectively. These represent the distances at which FRET efficiency is approximately 50%. Using the average measured diameter for carboxyl-modified and streptavidin-modified QDs, it is clear that the closest possible distance between the center of the QD and the quencher molecule would be 3.5 and 6.75 nm, respectively. From FRET efficiency calculations, distances greater than 5 nm from the center of this QD should result in less than 50% FRET efficiency. In fact, based on their measured diameter, it can be estimated that the carboxyl-modified QD–Iowa Black interaction would have a maximum FRET efficiency of 89% while the streptavidin-modified QD–Iowa Black interaction would have a maximum FRET efficiency of only 14%. Although these values would change relative to the exact number of quencher molecules associated with each QD, this demonstrates that FRET-based quenching of QDs is highly dependent upon the distance of the quencher from the QD. Therefore, the differences in between covalently-linked and streptavidin-linked QD molecular beacons could be dependent upon their initial quenched state. If the covalently-linked molecular beacons were initially better quenched than the streptavidin-linked molecular beacons, then hybridization-based unquenching would be more pronounced for the covalently-linked molecular beacons.

In addition to comparing linkage methods we also compared the effectiveness of different quencher moieties. We compared the dark quencher Iowa Black FQ with both dabcyl and 1.4 nm Nanogold particles. Dabcyl has been used previously to create QD molecular beacons through carboxyl linkage [9]. Other groups have used gold nanoparticles to quench QDs through a variety of attachment methods [19,24]. Gueroui and co-workers [19] demonstrated that modifying the spatial separation between 1.4 nm Nanogold and a 522 nm QD (Qdot Corporation) resulted in varying quenching efficiencies. At a distance of 10 nm, the QD was quenched 20% while distances of 7.5 and 5.9 nm quenched the QD 50% and 80%, respectively. For our study, we attached both dabcyl and Iowa Black FQ quenched molecular beacons to carboxyl-linked QDs and also attached 1.4 nm Nanogold molecular beacons to streptavidin-linked QDs. To directly compare these quenchers, we mixed the QD molecular

beacons with both complement and non-complement DNA and measured their fluorescent intensity (Fig. 3). These data showed that Iowa Black FQ molecular beacons yielded a maximum increase in fluorescence of 3.3-fold; however, dabcyl molecular beacons did not show any relative increase in fluorescence during target DNA hybridization. These data differ from a previous report by Kim et al. [9], where dabcyl was used for successful QD quenching. Kim and co-workers, however, used 490 nm QDs that have considerably better spectral overlap with the absorbance maximum of the dabcyl quencher. Therefore, the quenching efficiency of the dabcyl quencher for the 490 nm QDs used by Kim and co-workers is expected to be much higher. Fig. 5 shows the calculated spectral overlap of these QD–quencher pairs. As indicated in the spectra, Iowa Black FQ is a much better quencher for 525 nm QDs than dabcyl, due to the better overlap between the QD emission spectrum and quencher absorbance spectrum. The spectral overlap integral for the Iowa Black FQ–525 nm QD interaction was calculated to be $2.6 \times 10^{-13} \text{ cm}^{-1} \text{ M}^{-1}$, while that for the dabcyl–525 nm QD interaction was only $7.4 \times 10^{-14} \text{ cm}^{-1} \text{ M}^{-1}$. This indicates that the Iowa Black FQ quencher is a significantly better quencher than dabcyl when using 525 nm QDs.

For the 1.4 nm Nanogold quencher, we observed a 1.9-fold increase in fluorescence upon interaction with complementary DNA target. As described above, there may be some differences in quenching ability due to the linkage method used. Using the hydrodynamic radius measurements of the streptavidin-modified QDs, the minimum separation distance between the 1.4 nm Nanogold particle and the QD, is approximately 7.5 nm. Gueroui and co-workers [19] reported that, based on single molecule observations, a distance of 7.5 nm between a QD and Nanogold particle resulted in only 50% quenching efficiency. This corresponds well to our data, in which there was nearly a 2-fold increase in fluorescence intensity upon interaction with target DNA.

Finally, for any molecular sensing system, there must be specificity between target and non-target molecules. For each of the QD molecular beacons that were synthesized, specificity was determined by comparing hybridization with perfectly complementary DNA and non-complementary DNA. Regardless of the linkage method or the quencher moiety that was used, hybridization with complementary DNA yielded approximately 2-fold higher fluorescence intensity than hybridization with equal concentrations of non-complementary DNA. Dabcyl-quenched molecular beacons, however, did not exhibit changes in fluorescence for complementary or non-complementary DNA and the Nanogold-quenched beacons yielded only 1.6-fold higher intensity for complementary DNA. These data indicate significant specificity of the molecular beacons for the target DNA sequence and suggest that they could be used for discriminatory assays.

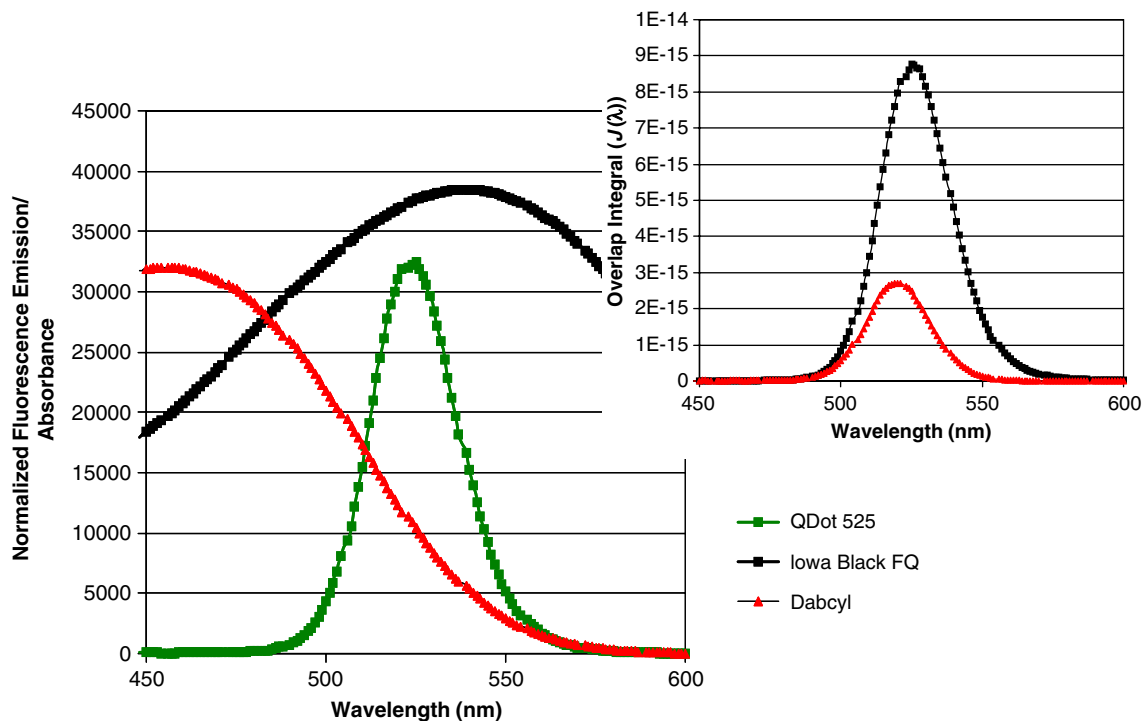


Fig. 5. Normalized spectra for 525 nm Qdots (grey squares), dabcyI (grey triangles) and Iowa Black FQ (black) and their overlap spectra (inset graph). The absorbance and emission spectra for the quenchers and Qdots, were normalized according to previous work by Medintz et al. [4]. The overlap integral ($J(\lambda)$) was calculated using these normalized data over a step size of 1 nm. For the inset graph, the overlap integral for dabcyI-Qdot 525 is shown in grey triangles, while the overlap integral for Iowa Black FQ-Qdot 525 is shown in black squares.

5. Conclusions

In summary, we have shown that QD molecular beacons are viable substrates for fluorescence-based DNA detection assays. We have also shown that a variety of attachment chemistries and quencher moieties can be used for QD molecular beacon synthesis. As expected, our data demonstrate that the spectral overlap of the QD fluorophore and the quencher directly affects the quenching efficiency of the molecular beacon. We have also shown that the QD to DNA linkage method has a direct effect on this efficiency. As with all FRET-based systems, the distance between the donor and acceptor must be tuned to provide the optimal efficiency for energy transfer. These data outline the importance of choosing proper linkage methods and quencher moieties for creating high quality QD molecular beacons.

Acknowledgements

Financial support was provided by USDA Grant #03-35201-13691, FDA Grant #06000002499A, and NIH Grant # 2004-DN-BX-K001. This work was performed in part at the Cornell NanoScale Science and Technology Facility (a member of the National Nanofabrication Users Network) which is supported by the National Science Foundation under Grant ECS-9731293, its users, Cornell University and Industrial Affiliates. This work also made use of STC shared experimental facilities supported by the National

Science Foundation under Agreement No. ECS-9876771 at the Cornell Nanobiotechnology Center.

References

- [1] Hohng S, Ha T. Single-molecule quantum-dot fluorescence resonance energy transfer. *Chem Phys Chem* 2005;6:956–60.
- [2] Willard DM, Van Orden A. Quantum dots: resonant energy-transfer sensor. *Nat Mater* 2003;2:575–6.
- [3] Han M, Gao X, Su JZ, Nie S. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat Biotechnol* 2001;19:631–5.
- [4] Medintz IL, Clapp AR, Mattoussi H, Goldman ER, Fisher B, Mauro JM. Self-assembled nanoscale biosensors based on quantum dot FRET donors. *Nat Mater* 2003;2:630–8.
- [5] Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. Quantum dot bioconjugates for imaging, labelling and sensing. *Nat Mater* 2005;4:435–46.
- [6] Liu X, Farmerie W, Schuster S, Tan W. Molecular beacons for DNA biosensors with micrometer to submicrometer dimensions. *Anal Biochem* 2000;283:56–63.
- [7] Robelek R, Niu L, Schmid EL, Knoll W. Multiplexed hybridization detection of quantum dot-conjugated DNA sequences using surface plasmon enhanced fluorescence microscopy and spectrometry. *Anal Chem* 2004;76:6160–5.
- [8] Epstein J, Biran I, Walt DR. Fluorescence-based nucleic acid detection and microarrays. *Anal Chim Acta* 2002;469:3–36.
- [9] Kim JH, Morikis D, Ozkan M. Adaptation of inorganic quantum dots for stable molecular beacons. *Sensors Actuators B* 2004;102:315–9.
- [10] Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol* 1996;14:303–8.

- [11] Tan L, Li Y, Drake TJ, Moroz L, Wang K, Li J, et al. Molecular beacons for bioanalytical applications. *Analyst* 2005;130:1002–5.
- [12] Vet JA, Marras SA. Design and optimization of molecular beacon real-time polymerase chain reaction assays. *Methods Mol Biol* 2005;288:273–90.
- [13] Tsourkas A, Behlke MA, Rose SD, Bao G. Hybridization kinetics and thermodynamics of molecular beacons. *Nucleic Acids Res* 2003;31:1319–30.
- [14] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403–10.
- [15] Hermanson GT. *Bioconjugate techniques*. New York: Academic Press; 1996.
- [17] Clapp AR, Medintz IL, Mauro JM, Fisher BR, Bawendi MG, Mattoussi H. Fluorescence resonance energy transfer between quantum dot donors and dye-labeled protein acceptors. *J Am Chem Soc* 2004;126:301–10.
- [18] Du H, Strohsahl CM, Camera J, Miller BL, Krauss TD. Sensitivity and specificity of metal surface-immobilized “molecular beacon” biosensors. *J Am Chem Soc* 2005;127:7932–40.
- [19] Gueroui Z, Libchaber A. Single-molecule measurements of gold-quenched quantum dots. *Phys Rev Lett* 2004;93:166108.
- [20] Dubertret B, Calame M, Libchaber AJ. Single-mismatch detection using gold-quenched fluorescent oligonucleotides. *Nat Biotechnol* 2001;19:365–70.
- [21] Venkateswarlu V, Manjunath K. Preparation, characterization and in vitro release kinetics of clozapine solid lipid nanoparticles. *J Control Rel* 2004;95:627–38.
- [22] O’Neal D, Harrip P, Dragicevic G, Rae D, Best JD. A comparison of LDL size determination using gradient gel electrophoresis and light-scattering methods. *J Lipid Res* 1998;39:2086–90.
- [23] Pazy Y, Laitinen OH, Ravoy B, Kulomaa MS, Wilchek M, Bayer EA, et al. Crystallization and preliminary X-ray analysis of W120 K mutant of streptavidin. *Acta Crystallogr D Biol Crystallogr* 2001;57:1885–6.
- [24] Dyadyusha L, Yin H, Jaiswal S, Brown T, Baumberg JJ, Booy FP, et al. Quenching of CdSe quantum dot emission, a new approach for biosensing. *Chem Commun (Cambridge)* 2005:3201–3.