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QUANTUM DOTS IN MOLECULAR DETECTION OF DISEASE

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ABSTRACT
The unique photophysical properties of semiconductor quantum dots (QDs) have made them ideal for use as spectral labels and luminescent probes. In this review, applications are presented in which QDs function as active participants in nanoscale biosensor assemblies, where replacing traditional molecular fluorophores results in improved assay performance. Specific focus is on disease detection with applications including multiplexed target detection, mutation detection by coincidence analysis and QD-based FRET reporters for miRNA detection and DNA methylation analysis.

INTRODUCTION

Quantum dots have high quantum yields, are highly photostable and allow continuous monitoring over extended periods of time. In recent years, there have been several QD applications that have utilized nanocrystals as scaffolds and active participants in biosensing, wherein biological specificity within hybrid inorganic/organic assemblies results in capture and detection of molecular disease markers. In these applications, the high surface area to volume ratio, and well-documented conjugation chemistries for QDs allow attachment of biomolecular probes, thus transforming the nanocrsytals into scaffolds for molecular interactions. Fluorescence Resonance Energy Transfer (FRET) has been widely used in molecular detection of disease due to the extreme sensitivity of FRET to the intermolecular distance between the donor and acceptor fluorophores. FRET has been widely used for various applications including characterizing structural properties and conformational changes of molecules. QDs make excellent FRET donors due to their broad absorption and narrow emission as well as size-tunable photoluminescence spectra. In addition, the broad excitation range for QDs allows the choice of an excitation wavelength in order to minimize the direct excitation of the acceptor.

In this report, signal transduction in DNA-QD biosensors is shown using three separate formats, including co-localization imaging, coincidence-based spectroscopy, and QD-FRET, where the QD serves as an energy donor with an organic fluorescent dye pair [3, 4]. In the first format, the distinct emission wavelengths and small physical size of the QDs enables multiplexed detection of three B. anthracis- related gene targets in the colocalization-imaging example. In the second format, using a highly sensitive spectroscopy platform, and the oligonucleotide ligation assay enables detection of Kras point mutations in the single molecule, two-color coincidence experiment. The distinct emission wavelengths proved useful in allowing efficient two-color coincidence analysis using a single excitation laser. Finally, in the third format, using QD-FRET reporters, the QD-oligonucleotide hybrid probes yield extremely low background fluorescence, enabling detection of low concentration targets. Detection of miRNA is first illustrated, where simple hybridization probes are ideal in capturing the short-stranded RNA species. In another application, QD-FRET is combined with methylation-specific PCR (MSP) for detection of picograms of methylated DNA in the presence of excess unmethylated alleles.

EXPERIMENTAL

-Multiplex Detection through Co-localization Analysis
A schematic for using QDs in multiplexed analysis of low abundant DNA targets is shown in Figure 1(a), top panel. Dual-color QD-conjugated oligonucleotide probes are hybridized with target DNA to form nanoassemblies [1]. Hybridized nanoassemblies appear as mixed color pixels, due to the diffraction limited resolution of the optical system, while the brightness of the QDs enable colorimetric measurements using a CCD (Figure 1(a), bottom panel). This method remains separation-free, as unassembled molecules pose no background signal, while the distinct emission spectra of QDs enables multiplexed analysis using several oligonucleotide probes for multiple color combinations.

-Mutation Detection via Coincidence Analysis
The schematic in Figure 1(b) shows how the oligonucleotide ligation assay (OLA) is coupled to the QD scaffold to encode mutation specific information within each nanoassembly. Briefly, two probes, a discrimination probe and a dye-labeled probe are enzymatically ligated together in the presence of a perfect match DNA target. Detection of the ligated products are then indicated by dual-color fluorescence coincident bursts as nanoassemblies are driven past a laser-induced fluorescence (LIF) spectroscopic system [2, 4, 5], using pressure-driven flow. In this case the broad absorption spectrum of the QD enables single laser-line excitation but dual-color emission, thus greatly simplifying instrumentation. Once again, the assay remains homogenous, this time due to the lack of enzymatic probe coupling in the absence of target molecules.

-Specific miRNA Detection via QD-FRET
DNA-QD is transformed into nanoassemblies in FRET reporters as shown in the schematic (Figure 1(c)). In this case, the nanoassemblies are formed through hybridization of two probes and a target molecule. A streptavidin-conjugated QD functions as both a target concentrator, for capture of multiple targets, and a FRET energy donor. Target molecules are sandwiched by the two probes, and then captured by the QD [4]. The

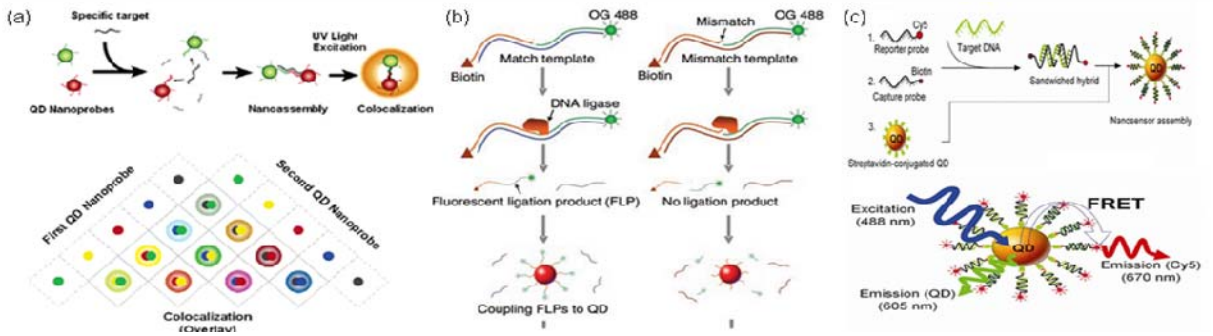

Figure 1 Schematic. (a) Nanoassembly formation using QD-conjugated oligonucleotides (upper). Distinct QD emission spectra enable multiplexing using several hybridization probes and multiple color combinations (lower) [1] (b). Process flow for nanoassembly formation in the presence of match templates using OLA (left). No nanoassemblies are formed in the presence of the mismatch templates (right). Coincident, single particle signal in both emission channels enable enumeration of ligated probes (left), while chance coincident bursts and ligation errors yield low background signal (right) [2] (c) Process flow for QD-FRET nanoassembly formation in the presence of target, resulting in multiple probe-target hybrids captured by single QD (upper). Excitation of QDs results in FRET through the organic dye within the reporter probe. In the absence of targets, the reporter probe remains separated from the QD donor (lower).[4]

resulting assembly brings the fluorophore from the reporter probe in close proximity to the QD donor. This results in fluorescence emission from acceptors, by means of FRET, upon QD excitation. Again, this assay remains homogenous and separation-free, as reporter and capture probes remain separated in the absence of target, preventing FRET signals.

-DNA Methylation Analysis via QD-FRET

The sensitivity of QD-FRET sensing is combined with the specificity of methylation-specific PCR (MSP) to detect DNA methylation. Briefly, bisulfite conversion of DNA renders only methylated targets available for PCR amplification using methylation- specific primers. These primers serve as the reporter and capture probes depicted in Figure 1(c), resulting in capture of methylation-specific PCR amplicons by QD donors. The resulting QD nanoassemblies are then analyzed using either bulk or single molecule methods. These methods serve to significantly reduce the quantity of sample necessary for analysis, while also allowing for quantification and decreasing post-amplification detection limits.

RESULTS

Three gene targets for bacillus anthracis were chosen to demonstrate the efficacy of multiplex detection via QD colocalization. This example is especially pertinent, as identification of rpoB, pagA, and capC, are all necessary to determine pathogenicity. Figure 2(a) shows how three QDs with distinct emission wavelengths, 525QD, 605QD, and 705QD, were combined with three pairs of target-specific DNA probes to perform colorimetric analysis on four separate DNA samples (Figure 2(b)).

The images in Figure 2(c) show that specific color combinations were observed only in multiple DNA target containing samples, yielding unambiguous detection of the pathogenic model (Image I). Up to 10 QDs have been demonstrated with distinguishable emission wavelengths; therefore, future applications of this combinatorial technique could allow simultaneous detection of up to 45 separate targets.

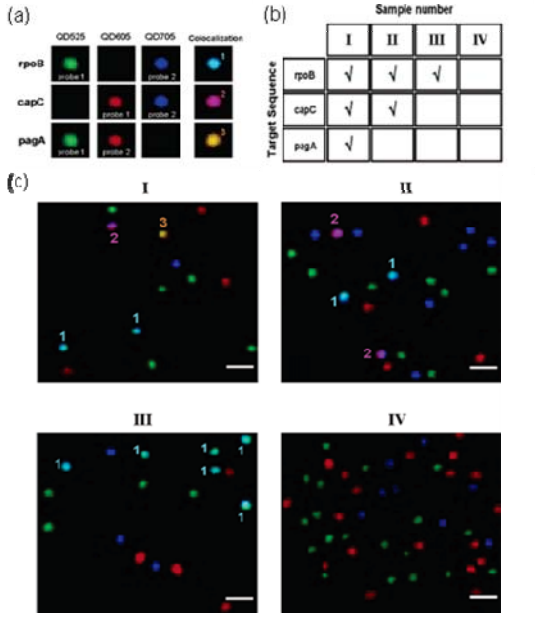

Figure 2 Multiplexed analysis of anthrax-related DNA targets [1]. (a) Color combinations for three pairs of target- specific QD nanoprobes. (b) Four samples used to detect different combinations of the three targets, rpoB, capC, and pagA. (c) Fluorescein images from those four samples showing multiplexed detection correlated with the mixtures from 2(b) (Bar dimension: 1μm).

Upon combining the point-mutation discerning oligonucleotide ligation assay with the highly-sensitive confocal microscope, allele discrimination is made possible. Figure 3(a) illustrates that in the absence of mutant targets the homozygous sample shows little coincident burst counts from the mutant probe. However, the heterozygous DNA sample reveals equal coincident bursts from both the wild-type and mutant probes. In addition, low error rates in the ligation assay and low probabilities for chance (non-assembled) coincident peaks yield low background coincidence rates and a point mutation selectivity factor of greater than 100,000 (Figure

3(b)). These results show an example of detecting Kras (codon 12 GGT to GTT) mutation within clinical samples from patients with ovarian serous borderline tumors. Importantly, utilization of the QD and its broad absorption spectra enables coincidence analysis using a single excitation source. This stands as a major simplification, compared to dual-excitation systems, which require careful laser alignments and delicate operational parameters.

Figure 4 shows that extension of the QD-nanoassembly scheme to QD-FRET. For this study, probe sequences were identical to those used in the study by Neely et. al [6]. As seen in Figure 4(a),(b), fluorescent bursts indicate donor probes in solutions containing target molecules. However, in the presence of non-specific targets (salmon sperm RNA) absence of bursts in the acceptor detector suggests that direct excitation of acceptors or leakage of donor emission into the acceptor was minimal (Figure 4(c)). In addition, the near-zero background acceptor fluorescence observed in this non-specific control suggests high-detection efficiency for the miRNA probes.

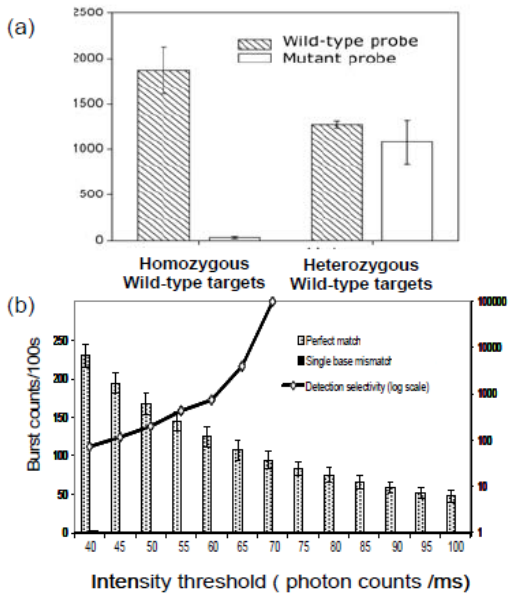

Figure 3 (a) Results from single particle coincident burst analysis from either homozygous or heterozygous DNA samples, using both wild-type and mutant probes [2].
(b) Coincident signal counts for perfect match and single-base (gray) and mismatch experiments (black) at different fluorescence thresholds. The allele discrimination selectivity factor is plotted in log scale (black line).

This is especially important as the small size of RNA make amplification protocols more difficult. In this example, the broad excitation of QDs allows particle excitation at a wavelength near the minimum of the Cy5 (acceptor) spectrum, greatly reducing interference from direct dye excitation.

In a separate study, we examined the benefit of this unique property of QDs in the sandwich assay through single particle counting experiments. For comparison we used a molecular beacon (MB) probe against the same DNA target [4]. Traditionally, MBs are restricted in non-amplification based assays by non-perfect fluorescence quenching and conformational fluctuations. Indeed, Figure 5 shows that, unlike MBs, QD-FRET probes yield low background signal over a range of probe concentrations, enabling extremely low detection limits. In this experiment, the QD-FRET reporter resulted in 100-fold decrease in the detection limit, as monitored by the confocal fluorescence spectroscopy platform. However, these extraordinary assay improvements need not be limited to amplification-free assays.

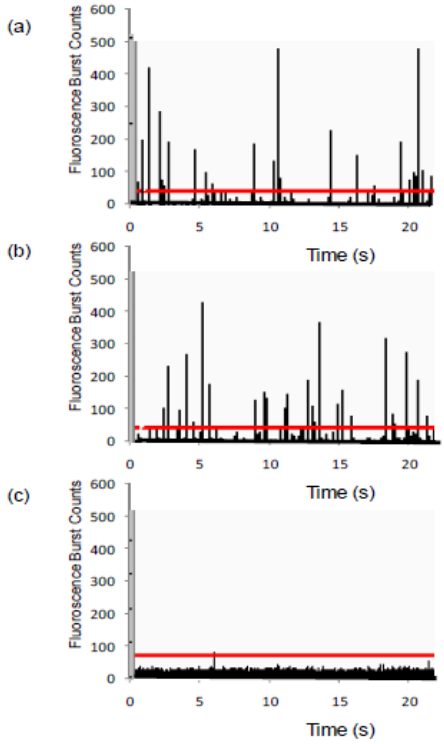

Figure 4 miRNA detection with QD-FRET nanoassemblies. (a) (b) Raw traces of the QD-FRET response to 1 and 10 pM target containing samples.,(c) Low occurrence background peaks from no target control samples.

QD-FRET sensing was extended to analyze DNA methylation [7]. In this case, DNA is amplified using PCR wherein the forward and reverse primers are labeled with a biotin and a fluorophore respectively. The resulting labeled-PCR product then concentrates around streptavidin functionalized QDs through streptavidin-biotin affinity. Upon suitably exciting the QD with a uv-light source, the nanoassembly formed allows for FRET to occur between the QD donor and the fluorophore acceptor. Using fewer cycles of PCR to quantify methylation at the log-linear phase of amplification, QD-FRET readout is used to analyze varying levels of DNA methylation of the gene promoter. As seen in Figure 6(a), the QD photoluminescence is increasingly quenched with increasing percent methylation. This corresponds with an increase in acceptor (Cy5) emission. Upon calculating the FRET efficiency based on the proximity ratio formalism, $E = I_A/(I_A+I_D)$, where I_D and I_A corresponding to donor and acceptor intensity, methylation levels of different samples

can be compared. Normalized FRET efficiency values are presented in Figure 6(b) and demonstrate the ability to accurately quantify DNA methylation levels.

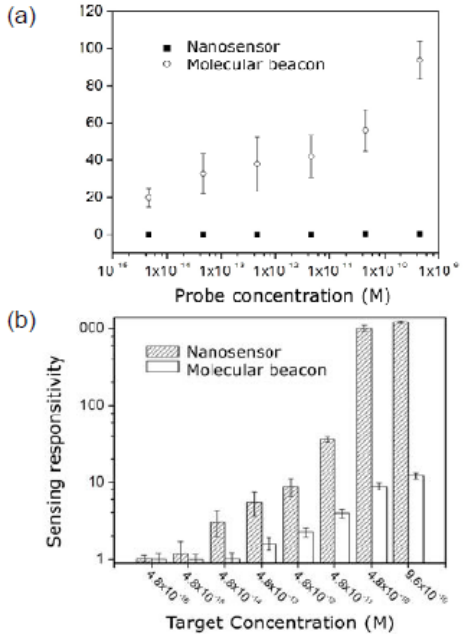

Figure 5 (a) Background fluorescence bursts as a function of probe concentration for both the QD-FRET and molecular beacon sensor [4]. (b) Sensor responsivity for different target concentrations of both the QD-FRET and molecular beacon (MB, reporter probe, and capture probe kept at 4.8 X 10^{-10} M concentrations).

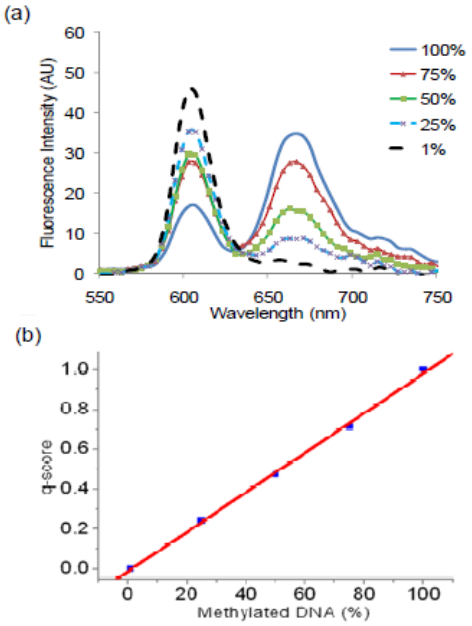

Figure 6 Ability to quantify DNA methylation using QD-FRET [7] (a) Different ratios of methylated: unmethylated DNA were used and analyzed using QD-FRET sensing. Increasing percent methylation levels are accompanied by an increase in acceptor (Cy5) emission at 670 nm and corresponding donor (QD605) quenching at 605 nm. (b) Normalized FRET efficiencies (q-scores) are plotted for the varying levels of promoter methylation and a linear fit is observed with $r^2 = 0.999$.

DISCUSSION AND CONCLUSION

This report shows several specific biosensing applications, where the unique properties of QDs give them distinct advantages over traditional fluorophores. In addition, these examples use the QD as an active participant or nanoscaffold, instead of merely emulating the traditional organic fluorophore-based techniques. In each of the applications, self-assembly of hybrid inorganic/organic components result in transduction of biological information pertaining to a disease state. Three separate detection strategies are utilized, including co-localization imaging, single particle coincident and FRET-mediated burst analysis. Molecule by molecule enumeration was shown in both amplification-free formats and in conjunction with the oligonucleotide ligation assay or polymerase chain reaction. Finally, two of these techniques were shown to be effective in probing the novel QD-FRET applications of specific miRNA counting and DNA methylation analysis. These distinctive results obtained using QD-based biomolecular probes confirm that use of these unique materials will continue to expand in the molecular detection of disease.

ACKNOWLEDGEMENTS

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