Quantum Dot-Mediated Separation-Free Assay for Point Mutation Detection

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ABSTRACT

By integrating a quantum dot-mediated biosensing technique, dual-color coincidence detection scheme and oligonucleotide ligation assay, we have developed a rapid point mutation detection platform in a separation-free format. Detection of the target signal is carried out on a single quantum dot basis using an ultrasensitive confocal fluorescence spectroscopic system. Comparing with other nanoparticle-based, separation-free assays with single base discrimination sensitivity, our method shows advantages in speed, simplicity, and sensitivity. Moreover, we have demonstrated that this quantum dot-based assay allows point mutation detection with nearly infinite specificity.

Keywords: quantum dots, coincidence detection, confocal spectroscopy, point mutation detection

1 INTRODUCTION

Recently, numerous techniques incorporating DNA-conjugated nanoparticles have been proposed for the detection of specific nucleic acid sequence with single base discrimination specificity. These approaches take advantage of material's property changes (optical [1-10], electrochemical [11-13], magnetic [14] or mechanical [15]) upon DNA hybridizations or enzyme-mediated reactions. Among them, the optical-based, separation-free assays have raised the greatest interest due to their simplicity, automation friendliness, and high analysis rate [16, 17]. More important, performing molecular reactions and detection in a homogeneous, separation-free format facilitates the binding kinetics [5, 18], thereby improving detection sensitivity.

Gold nanoparticle cross-linking aggregates with different interparticle distances will appear different colors due to surface plasmon resonance of the gold, rendering this a method for specific polynucleotide detection [1]. The sharp melting transitions of gold aggregates are used to differentiate a perfect match target from a strand with single base mismatch. The limitation of this approach is that it is inherently a one-color system based on gray scale [2]. Precise temperature control is also required during test. Another gold nanoparticle aggregation system induced by non-cross-linking DNA hybridization was also reported [6, 7, 9]. It was found that single- and double- stranded oligonucleotides gave distinct nanoparticle aggregation phenomena upon adding salt to the solutions. Nanoparticle aggregation was measured with a UV-visible spectrophotometer at bulk level, making this method less quantitative in low-abundant mutation detection. Gold nanoparticles were also used as quenchers in homogeneous fluorescence resonance energy transfer (FRET) assays [4, 8]. Nevertheless, the preparation of oligonucleotide-modified gold nanoparticles is time consuming, typically from tens of hours to a few days [7].

For today's clinical application, assays for specific nucleic acid detection with single base discrimination specificity and short sample preparation time, but without the need of temperature control during test, complicated probe designs, and separation steps, are highly desired. Here we report a quantum dot (QD)-mediated biosensing technique that fulfills those goals. QDs (2-10 nm), such as CdSe-ZnS core-shell nanocrystals, have size-dependent tunable photo-luminescence, broad excitation and narrow emission bandwidths, as well as high quantum efficiency and photostability [3, 10, 19]. They can be surface-functionalized with different probe molecules (e.g. oligonucleotides, peptides, and antibodies), facilitating the detections of different biomolecules including DNA, RNA, and proteins. By using confocal fluorescence spectroscopic system, QDs can be analyzed at a single dot level, making this QD-based assay a good way for quantitative analysis of genomic variants.

2 PRINCIPLE OF DETECTION

A schematic representation of the QD-mediated biosensing assay and coincidence detection scheme is illustrated in Figure 1. First, a biotin-conjugated discrimination primer and an Oregon Green 488 (OG488, Molecular Probes)-labeled reporter primer are covalently linked by DNA ligase using a perfect match target as template (Fig. 1A, left) [20]. After denaturing the duplexes by heat, the fluorescent ligation product (FLP), which is biotinylated at one terminus and OG488-labeled at the other, is separated from the template. Streptavidin coated QDs, which serve as nano-scaffolds, are added to the solution to capture the FLPs through biotin-streptavidin interaction. Whenever a QD-FLPs nanoassembly flows through the miniscule detection volume of the confocal spectroscopic system (Figure 1B), QD and OG488 dyes are simultaneously excited and the resulting photon emissions are separately and simultaneously detected by QD and OG488 detection channels (Figure 1C, left). These simultaneous burst signals are termed coincident signals

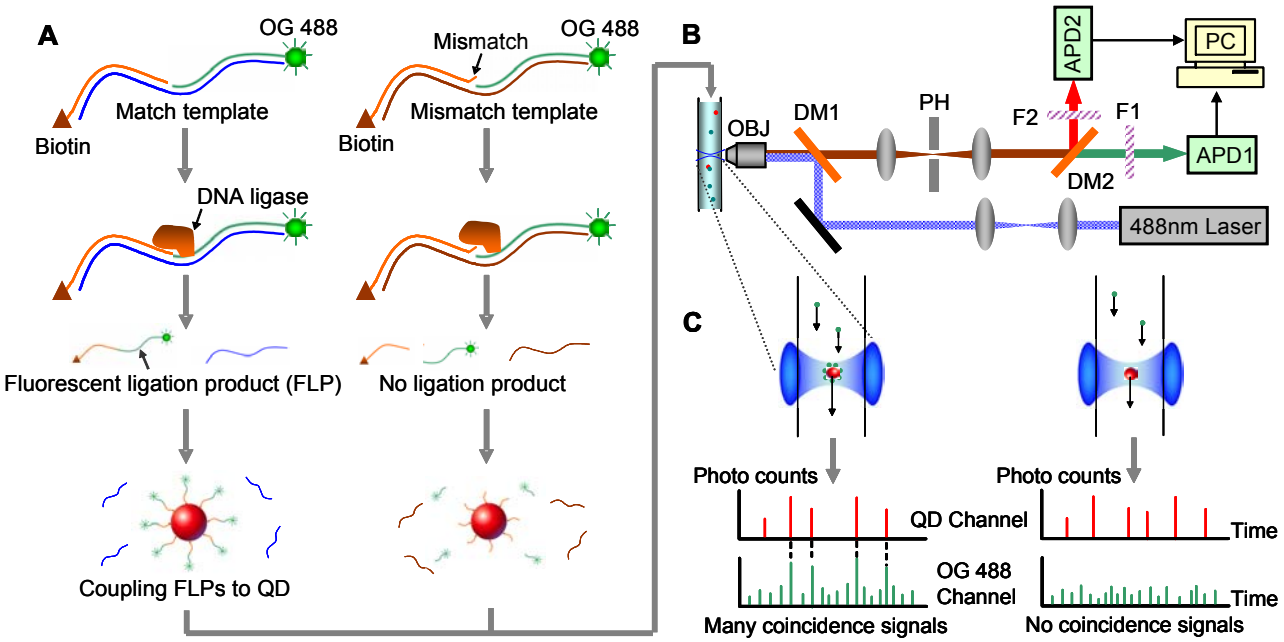

Figure 1. Schematic concept of QD-mediated biosensing assay and coincidence analysis scheme (A) Oligonucleotide ligation assay. (B) Confocal fluorescence spectroscopic system. (C) Coincidence detection: the coincident signals (marked with dash lines) serve as indicators of perfect match targets.

and each pair of coincident signals is one coincidence event, which in fact represents the passage of one QD-FLPs nanoassembly. On the other hand, ligation does not occur and FLPs are not formed when single base mismatch targets are used as templates (Figure 1A, right). No coincident signals are seen during test (Figure 1C, right). Therefore, the coincident signals can be used to distinguish perfect match targets from single base mismatch targets.

3 EXPERIMENTS

3.1 Sample Preparation

For human β-globin gene point mutation detection, a 20 µl reaction mixture comprised of 2.4 picomoles of reporter primers (pAGGAGAAGTCTGCCGT-OG488), 2.4 picomoles of discrimination primers (Biotin-GTGCACCTGACTCCTG), 2.4 picomoles of targets (ACGGCAGACTTCTCCTCAGGAGTCAGGTGCA C or ACGGCAGACTTCTCCTGAGGAGTCAGG TGCAC), 1 unit of T4 DNA ligase, and 1× company supplied T4 ligation buffer (New England BioLabs) was prepared. After carrying out ligation at 22 °C for half an hour, 1 µl aliquots were removed from ligation solutions and were diluted with 98 µl PBS buffer (10 mM sodium phosphate, 100 mM NaCl, pH 7.0) in a test tube. The test tube was placed in an 85 °C hot water bath for 5 min to stop the ligation and to completely denature primer/template duplexes, following by dipping in ice water bath for 5 min. The tube was then stored at 4 °C refrigerator until required for the QD coupling reaction.

CdSe-ZnS QDs (Quantum Dot Corp.) with peak emission wavelength at 605nm were used in this report. These QDs have been conjugated with streptavidin through a carbodiimide-mediated coupling reaction, giving 15~25 streptavidins on each QD. QDs were first diluted with PBS buffer to a concentration of 4 nM. One microliter aliquots of QD solution were then added to test tubes containing 99µl diluted and heat denatured ligation solution, resulting in a molar ratio of 30 to 1 between primers and QDs. The incubation took 20 min at room temperature, with mild agitation only at the beginning to quickly dispense QDs in solution. The QD coupling solutions were further diluted 10× prior to detection.

3.2 Fluorescence Detection

A single wavelength-excitation, dual-emission confocal spectroscopic system was used for fluorescence detection (Figure 1 (B)). A 488 nm argon laser was used as an excitation light source. A 100× 1.3 N.A. oil immersion apochromatic objective (OBJ) was used to focus the laser beam inside a 100 µm wide microcapillary. The emitted fluorescence signal was collected by the same objective. Dichroic mirror 1 (DM1) allowed light of wavelengths longer than 505 nm to pass through. A 50 µm pin hole (PH) was used to reject out of focus light, thus enhancing the signal-to-noise ratio. The detection volume of the confocal spectroscopic system was estimated to be ~ 1.5 fl. Dichroic

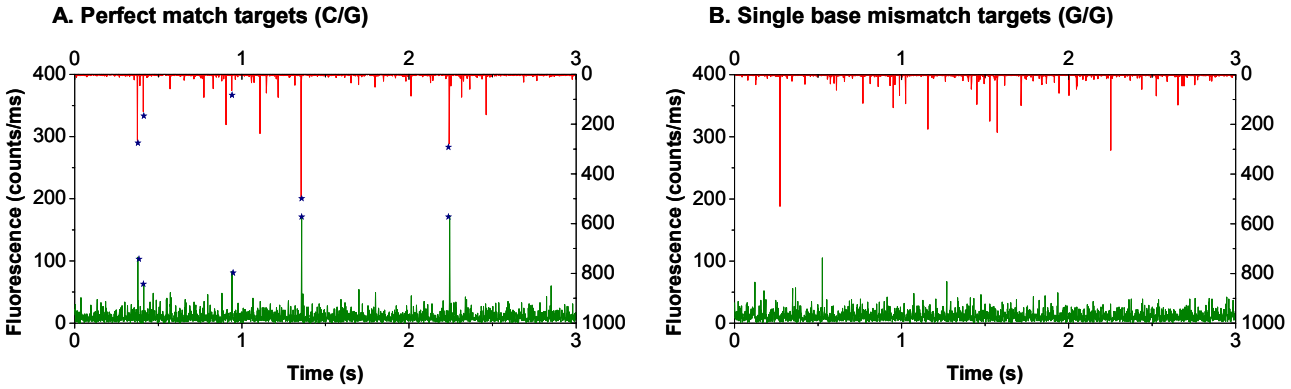

Figure 2. Raw fluorescence burst signals. Each burst represents the passage of one entity, which can be a QD (red bursts, upper), a reporter probe (green bursts, lower), or a QD-FLPs assembly (coincident signals, marked with asterisks). The data was binned in 1ms and thresholds were set to be 50 photon counts/ms for both OG488 and QD.

mirror 2 (DM2) allowed light of wavelengths longer than 565nm to pass through. Two avalanche photodiodes (APD1&2) were incorporated to register the two filtered emission wavelengths (524 nm for OG488 dyes and 605 nm for QDs). A digital counter and a program written in LabView were implemented to perform data acquisition and data analysis. The excitation laser power was kept at 40 μW at all time. Throughout the fluorescence detection, a small volume of sample (~ 5 μl) introduced to microcapillary was driven via hydrodynamic pumping at a flow rate of 1 μl/min.

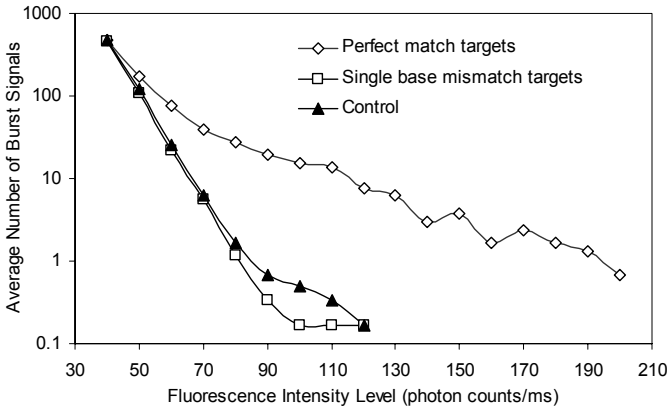

Figure 3. Fluorescence burst signal histogram indicates the OG488 fluorescence signals are greatly enhanced for those dyes bound to QDs.

4 RESULTS AND DISCUSSION

4.1 Detection of Single Base Variations in Oligonucleotide Targets

To reduce the effects of variability between DNA samples in evaluation of the assay, we began by analyzing single base variations in synthetic oligonucleotide targets which were derived from variants of human β-globin gene sequences. Figure 2A shows a representative trace of fluorescence signals measured from the sample containing perfect match targets. The coincident signals, marked with asterisks, detected in both channels evidence the formation of QD-FLPs nanoassemblies. In our confocal spectroscopic system, the analyte is measured within a stationary femtoliter detection volume, giving an extremely low background noise. Consequently, photon bursts emitted from single nanoassemblies could be effectively distinguished from background. In contrast, coincident signals were barely detected from the sample containing single base mismatch targets, suggesting the formation of nanoassemblies being prevented due to the lack of FLPs (Figure 2B).

4.2 Signal Amplification Due to Local Concentration of Organic Dyes

To address the effect of target signal amplification induced by QD as nano-scaffolds, we compared the intensity histograms of OG488 photon bursts detected in the perfect match experiment, the single base mismatch experiment, and an additional control experiment (Figure 3). The control experiment was conducted by measuring a sample containing only targets and ligation primers, but no T4 DNA ligase. Since ligation was prevented in this experiment, the fluorescent bursts detected in the OG488 channel should come from (1) the free reporter primers, (2) the undenatured primer/template sandwich structures, or (3) the impurities in the solution. As shown in Figure 3, the OG488 photon bursts detected in the control experiment had an intensity level typically lower than 100 photon counts/ms. A similar result was also observed in the mismatch experiment. In contrast, photon bursts with intensity as high as 200 photon counts/ms or higher were detected in the perfect match experiment. Nearly all these high intensity photon bursts detected in the perfect match experiment were found to be associated with coincidence events, evidencing signal amplification effect through the formation of QD-FLPs nanoassemblies. This amplification effect greatly facilitates the differentiation between the binding-induced target coincident signals and the stochastic

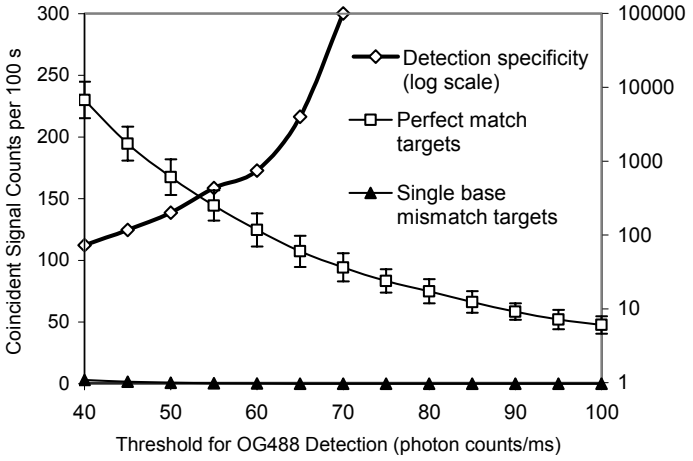

Figure 4. Average coincident signal counts per 100 s based on different threshold settings for OG488 detection. Error bars represent the standard deviations of six consecutive measurements.

background coincident signals. Also, unspecific photon bursts from impurities were filtered out using coincidence analysis. These results show that this QD-based biosensing assay is capable of detecting point mutations with nearly infinite specificity (Figure 4).

5 SUMMARY

In this report, we demonstrate that the organic fluorophores and semiconductor QDs can be linked to create a novel nanobiosensor. QD functions as a nanoscaffold which confines multiple FLPs in a nanoscale domain, leading to a high local concentration of organic fluorophores. Currently, we estimate that our nanobiosensor requires only ~ 30 organic fluorophores coupled to each QD to make a successful coincidence detection. This may open a new way of performing low abundant point mutation detection in separation-free format. In addition, QDs' extraordinary photophysical properties such as large Stokes' shift, broad absorption, and narrow emission spectra allow the selection of various organic fluorophores and QDs to make different nanobiosensor combinations, greatly facilitating multiplexing detections. Finally, this QD-mediated biosensing assay and coincidence detection scheme can serve as a universal detection platform, to which other enzyme-based techniques used to identify known polymorphisms and mutations such as single base extension (SBE) can also be incorporated.

6 ACKNOWLEDGEMEMT

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