Biosensors and Bioelectronics 26 (2011) 3346–3352

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A quantum dot-aptamer beacon using a DNA intercalating dye as the FRET reporter: Application to label-free thrombin detection

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ARTICLE INFO

Article history:
Received 1 November 2010
Received in revised form 9 January 2011
Accepted 13 January 2011
Available online 20 January 2011

Keywords:
Aptamer beacon
BOBO-3
Quadruplex
Quantum dot
Thrombin

ABSTRACT

A new quantum dot (QD)-aptamer (apt) beacon that acts by folding-induced dissociation of a DNA intercalating dye, BOBO-3(B), is demonstrated with label-free thrombin detection. The beacon, denoted as QD-apt:B, is constructed by (1) coupling of a single-stranded thrombin aptamer to Qdot 565 via EDC/Sulfo-NHS chemistry and (2) staining the duplex regions of the aptamer on QD with excess BOBO-3 before thrombin binding. When mixing a thrombin sample with QD-apt:B, BOBO-3 is competed away from the beacon due to target-induced aptamer folding, which then causes a decrease in QD fluorescence resonance energy transfer (FRET)-mediated BOBO-3 emission and achieves thrombin quantitation. In this work, the effects of Mg^{2+}, coupling time, and aptamer type on the beacon's performances are investigated and discussed thoroughly with various methods, including transmission electron microscopy (TEM), dynamic light scattering (DLS), and two-color differential gel electrophoresis. Using the best aptamer beacon (HTQ37), we attain highly specific and wide-range detection (from nM to μM) of thrombin in buffer, and the beacon can sense nM-range thrombin in 15% diluted serum. Compared to the reported QD aptamer assays, our method is advantageous from the aspect of using a simple sensory unit design without losing the detection sensitivity. Therefore, we consider the QD-apt:B beacon a potential alternative to immuno-reagents and an effective tool to study nucleic acid folding on QD as well.

1. Introduction

Rivaling antibodies and retaining structural and synthetic advantages, aptamers generated from *in vitro* selection, have been proven as an emerging class of functional oligonucleotides for highly specific and sensitive protein detection (Tombelli et al., 2005; Mairal et al., 2008). To date, a variety of aptamer-based alternatives to traditional immunoassays have been successfully demonstrated, such as the molecular beacon assay (Hamaguchi et al., 2001; Li et al., 2002), surface plasmon resonance detection (Li et al., 2007a), electrochemical methods (Xiao et al., 2005; Wang et al., 2009), microarray assay (Lao et al., 2009), and so on. Among these, the beacon assay is ideally suited for both *in vitro* and *in vivo* real-time and label-free protein sensing and relies on an aptamer probe with a labeled fluorescent donor at one end and an acceptor, either a fluorophore or a quencher, at the other end (Tan et al., 2004). When detecting the target, a binding-induced conformational change (e.g., from a stem-loop to a quadruplex) of aptamer occurs (Hamaguchi et al., 2001; Li et al., 2002; Xiao et al., 2005; Wang et al., 2009) and alters beacon's donor–acceptor distance, which leads to a change in the fluorescence resonance energy transfer (FRET) between the donor and the acceptor. Then the target binding can be quantitated by monitoring the emission change at either donor's or acceptor's characteristic wavelength or both. Thus, an aptamer beacon has not only fluorescence sensitivity but also multi-dimensional specificity for label-free protein detection.

Quantum dots (QDs) are core-shell assemblies of semiconductor nanoparticles that behave like fluorophores with tunable colors and sharp emission bands owing to the quantum confinement effect (Bruchez et al., 1998; Gill et al., 2008; Reiss et al., 2009). In the past decade, they have been demonstrated as promising rivals for traditional fluorescent dyes because of their broader excitation bands, larger effective Stokes' shifts, lower susceptibilities to photo-bleaching, and allowing multicolor parallel detection excited at a single UV light source. These niches have paved a new way for the development of QD-aptamer (QD-apt) beacons that use QDs to replace fluorescence molecules (e.g., fluorescein in Hamaguchi et al., 2001) as donors. The first QD-apt beacon reported by Levy et al. (2005) was a two-piece beacon construct consisted of a 5′-biotinylated anti-thrombin aptamer conjugated to streptavidin-coated Qdot 525 and an additional 3′ quencher-labeled antisense oligonucleotide that hybridized to and disrupted the aptamer structure. The binding of thrombin induced aptamer's

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doi:10.1016/j.bios.2011.01.015

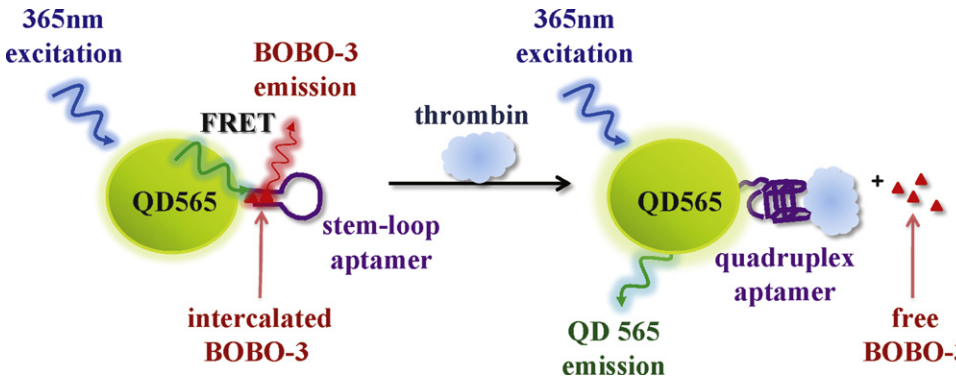

Fig. 1. Label-free detection of thrombin with a novel QD-apt:B beacon. Thrombin binding induces the conformational change of the aptamer on QD and then causes the dissociation of the stained BOBO-3 from QD-apt. Thus, the thrombin binding can be quantified by the decrease of QD FRET-mediated BOBO-3 emission. (*Note*: This figure is illustrated for simplicity, whereas multiple aptamer probes can be conjugated to each QD.)

folding into a tertiary quadruplex structure and thus competed away the quencher-labeled antisense from QD-apt, which then caused an increase in Qdot 525 emission. Finally, the researchers demonstrated a specific fluorescence response for real-time detection of unlabeled thrombin at 37 °C. After this inspiring work, some QD-apt beacon designs were reported to tackle label-free detection of thrombin (Swain et al., 2008), platelet derived growth factor (PDGF) (Kim et al., 2009), epithelial tumor marker Mucin 1 (Cheng et al., 2009), cocaine (Zhang and Johnson, 2009), respectively. Despite the difference in signaling and reporter designs (see Figure S1 in Supplementary Material), all of the reported QD-apt beacons were either two-piece or three-piece constructs that formed stable duplexes on QD prior to target binding and all functioned upon the target-induced strand displacement that caused a change in FRET for target quantitation.

The "multi-piece" QD-apt beacon designs required additional reporter-labeled oligonucleotides to work with the aptamer probe. In addition, forming a double-stranded DNA (dsDNA) recognition element from the GC-rich aptamer and its antisense prior to or after coupling to QD was required. Besides, the dsDNA might dissociate in the presence of a complementary genomic contaminant and led to a false QD FRET response (Levy et al., 2005). These drawbacks have driven us to develop a simple, "single-piece" QD-apt beacon (i.e., ssDNA-conjugated QD) that is competitive and cost-effective in clinical protein assays. Use of a single-piece QD beacon for detecting a DNA target was already achieved (Kim et al., 2004), but it has not been reported to date that a ssDNA aptamer-QD beacon construct is capable of label-free protein detection. In this work, a new, single-piece QD-apt beacon is developed with the aid of a dimeric cyanine DNA intercalating dye BOBO-3, 1,1′-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-pyridinium tetraiodide, (denoted as B; peak excitation at 570 nm; peak emission at 602 nm) (Lim et al., 2008) and is successfully proven with label-free thrombin detection. Our design is illustrated in Fig. 1. A single-stranded anti-thrombin aptamer probe, requiring neither fluorophore nor quencher end-labeling, is covalently conjugated to Qdot 565 (peak emission at 565 nm). Then BOBO-3 that shows large fluorescence enhancement when it is intercalated into a double helix (Ruedas-Rama et al., 2010a), is used to stain the duplex regions of the unfolded aptamer on QD to result in the QD-apt:B beacon. (*Note*: dash and colon in QD-apt:B stand for a covalent linkage and affinity adsorption, respectively.) Before thrombin binding, a FRET-mediated emission of the stained BOBO-3 can be observed while QD is illuminated at 365 nm. When thrombin binds to aptamer, the induced conformational change, from a stem-loop to a quadruplex (Hamaguchi et al., 2001; Wang et al., 2009), dissociates BOBO-3 from QD-apt. Thus, a decrease in the FRET-mediated BOBO-3 emission is observed and can be used to quantify of the thrombin concentration. This approach is majorly advantageous over the reported methods in the aspect of simplicity to generate the fluorescence change from QD-apt conjugates in a target specific manner. In addition, a limit of detection (LOD) of 1 nM thrombin has been achieved in this work, which is among the best detection limits reported for QD-aptamer based assays (cf. 1 μM LOD for thrombin in Levy et al., 2005; *ca.* 1 nM LOD for thrombin in Swain et al., 2008; 0.4 nM LOD for PDGF in Kim et al., 2009; 250 nM LOD for Mucin 1 in Cheng et al., 2009; 0.5 μM LOD for Cocaine in Zhang and Johnson, 2009, as illustrated in Figure S1 in Supplementary Material). Moreover, protein detection in the presence of diluted serum is also demonstrated.

2. Experimental

2.1. Preparation of QD-aptamer nanoconjugates

The QD-apt nanoconjugates were prepared by conjugating 5′ amine-modified aptamer probes (NH_2-apt; apt = HTQ20, HTQ37, and HTDQ29, refer to Supplementary Material for preparation of aptamer probes) to commercial carboxyl-coated Qdot 565 (QD-COOH, Qdot® ITK™ carboxyl quantum dots, Invitrogen, Carlsbad, CA, USA) through the EDC/Sulfo-NHS chemistry (Pereira and Lai, 2008) as described as follows.

$$\text{QD-COOH} + \text{NH}_2\text{-apt} \xrightarrow{\text{EDC/Sulfo-NHS}} \text{QD-apt} \quad (1)$$

The 1-ethyl-3-(3-dimethylamino propyl)carbodiimide (EDC) and *N*-hydroxysulfo-succinimide (Sulfo-NHS) reagents (Sigma–Aldrich, St. Louis, MO, USA) were used together to enhance the conjugation efficiency. For each QD-apt preparation, 0.5 nmol QD-COOH was first activated by mixing with 1 μmol EDC and 0.5 μmol Sulfo-NHS in phosphate-buffered saline (PBS: 10 mM phosphate, pH 7.4, 137 mM NaCl, and 3 mM KCl) for 15 min, and then 5 nmol NH_2-apt was added to the mixture for conjugation with different coupling time. To block the unreacted carboxyl sites on the QD surface, 0.4 μmol of ethanol amine was added to the mixture and incubated for an additional 1.5 h. After conjugation, additional 300 μL PBS was added to make up a 500-μL mixture for subsequent QD-apt purification with a 50 kD molecular weight cut-off centrifugal filter (Amicon Ultra-0.5, Millipore Corp., Billeria, MA, USA) that removed uncoupled NH_2-apt and excess chemical reagents. After five centrifugal purifications, the QD-apt products were harvested, added with PBS to 100 μl and then stored at 4 °C prior to use.