PAPER

Enzyme-free colorimetric detection systems based on the DNA strand displacement competition reaction

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Abstract

The strand displacement competition reaction assay is based on the dynamic equilibrium of the competitive hybridization of two oligonucleotides (A and B) to a third oligonucleotide (S). In the presence of an analyte that binds to a specific affinity-moiety conjugated to strand B, the equilibrium shifts, which can be detected by a shift in the fluorescence resonance energy transfer signal between dyes attached to the DNA strands. In the present study we have integrated an ATP aptamer in the strand B and demonstrated the optical detection of ATP. Furthermore we explore a new readout method using a split G-quadruplex DNAzyme for colorimetric readout of the detection of streptavidin by the naked eye. Finally, we integrate the whole G-quadruplex DNAzyme system in a single DNA strand and show that it is applicable to colorimetric detection.

Introduction

Thanks to its programmability [1], rich conjugation chemistry [2], and relatively high stability, DNA has great potential for development of sensors for species other than nucleic acids in biomedical diagnostics, on-site analysis, and point-of-care testing [3, 4]. Particularly, DNA-based homogeneous assays are simple, easy-to-perform, and obviates the need for time-consuming immobilization and washing, which are nevertheless common steps in most conventional heterogeneous assays [5]. The toehold-mediated DNA strand displacement competition (SDC) reaction was reported by Zhang et al for detection of single point mutations [6–8], and recently we have extended this to a sensor for other species [9]. Nanomolar detection of several small molecules and their respective antibodies/proteins with high sensitivity and specificity was demonstrated.

The assay includes two essential components: target recognition by a small molecule ligand conjugated on one of the oligonucleotide strands, and a signal transducer in the form of optical detection through fluorescence resonance energy transfer (FRET) provided by a donor-acceptor pair. Importantly, both of the components can be replaced by other modules. For example, aptamers can rival antibodies in terms of binding affinity, and have thus been used for target recognition for developing homogeneous assays in a solution phase [10–12]. For the signal transduction DNA G-quadruplexes, which were found to have the ability to enhance hemin peroxidation [13], can serve as detection labels that amplify sensing signals [14]. In the present study, we exemplify the incorporation of the two above-mentioned elements into our SDC assay, to expand the design flexibility and potential target variety of this system. More specifically, the toehold region [15] of one of the two competition strands was replaced by an Adenosine 5' triphosphate (ATP) aptamer [16], allowing structural-switching signaling upon target binding [17]. In another case, split G-quadruplex peroxidase probes [18] were extended from the ends of a hybridization pair, so that the equilibrium shifts of the SDC reaction can be readout from a color change of the solution. Finally, we compacted the three-strand system into one strand, and still achieved colorimetric detection of a protein target.

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Methods

Materials
All the amine-modified and unmodified oligonucleotides were purchased from DNA Technology A/S (Denmark) or Sigma-Aldrich (St. Louis, MO). The concentration of each oligonucleotide was measured by a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, MA).

Alexa Fluor® succinimidyl esters (647, 555 and 488) were purchased from Invitrogen (Carlsbad, CA). ATP was purchased from New England Biolabs (Ipswich, MA). All the other reagents were purchased from Sigma-Aldrich.

Oligonucleotides modified with Alexa dye or biotin were produced and purified as described previously [9]. In brief, the amine-modified oligonucleotides were conjugated with the NHS ester modified molecules by amide formation, followed by RP-HPLC (Agilent, CA) purification.

Absorbance at 420 nm was also measured by a Nanodrop 1000 spectrophotometer (UV–vis mode; Hi Abs).

Construction of the aptamer-based SDC assay for ATP detection
0.5 ml BILATEC tubes (Sigma-Aldrich) were used for all the reactions. The SDC assay was prepared by mixing DNA strands A, B and S variants in equal stoichiometric ratio (50 nM), in 1 x TAE/Mg\(^{2+}\) buffer pH 8. The target (ATP: 0.5 mM) or control (GTP: 1 mM; STV: 0.3 \(\mu\)M) molecules were added at the same time. Then the whole mixture was incubated at room temperature (RT) overnight in the dark. Since this method is based on thermodynamics instead of kinetics, the order of addition of different components is irrelevant to the final readout. All the concentrations specified here are the final concentration of each component in an assay of 70 \(\mu\)l.

FRET experiments
65 \(\mu\)l of the assay mixture was pipetted into a 60 \(\mu\)l quartz cuvette (Hellma Analytics), which was washed with 1 x TAE/Mg\(^{2+}\) three times between different samples. Fluorescence measurements were performed using a scanning spectrophotometer (Fluro-Max-3, HORIBA Jobin Yvon Inc.), with an integration time of 0.5 s, 5 nm slits and 1 nm wavelength intervals at 25 °C.

Samples were excited at 550 nm and fluorescence spectra arising from the FRET pair Alexa555 and Alexa647 were detected. Relative FRET efficiencies were calculated as \(E_r = \frac{I_{DA}}{I_{DA} + I_{DD}}\), where \(I_{DA}\) is the acceptor peak emission intensity at 667 nm and \(I_{DD}\) is the donor peak emission intensity at 567 nm. The experiments were repeated with five batches (table S2).

Construction of the SDC assay with split G-quadruplex peroxidase for Streptavidin (STV) detection
The SDC assay was prepared by mixing DNA strands A, B and S variants in equal stoichiometric ratio (200 nM), in 1 x TAE/Mg\(^{2+}\) buffer pH 7. STV (400 nM) or an equal volume of ddH\(_2\)O was also added at the same time. After incubation at RT for 3 h, hemin (2 \(\mu\)M), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (4 mM) and H\(_2\)O\(_2\) (4 mM) were added into the SDC assay mixture, followed by another 0.5 h incubation at RT in the dark before absorbance measurement orphotographing.

In the case of the one-strand system, the conditions and concentrations are the same as described above, except that there was only one DNA strand (L) in the assay. All experiments were performed at least three times, and each time the same trend was observed. All the concentrations specified here are the final concentrations of each component in an assay of 50 \(\mu\)l.

Results

ATP detection by aptamer-based SDC assay
The detection system consists of three strands A, B and S of which A and B compete about binding to S (figure 1). In the absence of target molecules, strand S prefers to hybridize with B, since B has a longer toehold than A (8nt blue versus 3nt red duplexes regions). In other words, because strands A, B and S have equal stoichiometry, there will be more duplex of BS than AS in the solution. The blue part of strand B is an ATP aptamer and in the presence of ATP folds into the aptamer structure where the toehold on B is a necessary part of the aptamer for target-binding. The 8nt toehold region of B is no longer available for binding to S, and therefore the equilibrium shifts more towards the AS duplex since it now forms the longer duplex. The scheme of this process is illustrated in figure 1(a).

A fluorophore pair is conjugated to strand A and S respectively, so that the FRET signal is linked to the population of AS. It is expected that the presence of ATP will lead to aptamer binding and formation of a larger population of AS. This will in turn lead to an increased FRET signal. Indeed, it is observed that only ATP can
cause the increase of FRET efficiency, while other control molecules such as guanosine 5′-triphosphate (GTP) do not have such an effect (figure 1(b), see raw fluorescence data in supplementary table S2). The decrease of the BS duplex population at the same time is confirmed in a three-dye system where strand B also has a fluorophore (supporting information figure S1). A titration curve is acquired and shown in supporting information figure S2 showing detection of ATP down to approximately 100 nM.

Colorimetric detection of STV by the SDC assay with split G-quadruplex peroxidase

When iron(III)-protoporphyrin (hemin) is present as a cofactor, DNA containing four or higher tracts of guanines can form horseradish peroxidase mimicking DNAzymes, which effectively catalyze the H₂O₂-mediated oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) into its radical form (ABTS−) with an obvious color change due to its absorption at 420 nm [13, 19]. The past decade witnessed an explosion of the use of G-quadruplexes peroxidase as an amplifying color reporter for the detection of nucleic acids [14] and other molecules [20], including proteins, small molecules, metal ions [21], etc. In addition to the general advantages of colorimetric sensor such as visibility to naked eyes, high sensitivity and low cost, DNAzymes received special attention because they can be stored as lyophilized powders at ambient temperature, and they can be reconstituted into active enzymes by simply dissolving in buffer.

Here we employed a split G-quadruplex strategy [18, 22, 23] in the signal output module of our SDC assay. The G-quadruplex was split into two parts, each lacking peroxidase activity by itself. One part possessed one GGG repeat, and was extended from the 5′ end of strand A. The other part possessed three GGG repeats and was extended from the 3′ end of strand S. The asymmetric split mode was reported to have lower background noise and more efficient reconstitution than symmetric probes [23]. When A hybridized with S, the full G-quadruplex structure as well as its peroxidase activity would be restored. Therefore, the extent of color change caused by increased formation of (ABTS−) reflects the fraction of duplex AS in the sample.

As a model system, we conjugated biotin to strand B for STV detection. The toehold regions of A and B are 2 and 4 nt respectively. Initially, S prefers to hybridize with B because BS has a higher melting temperature (Tm) than AS [9]. Upon binding of STV, the Tm of the BS decreases, resulting in a shift of the equilibrium toward AS (figure 2(a)). After half an hour in the presence of hemin, H₂O₂ and ABTS the effect of STV binding is observed as an increase of the absorption of the solution at 420 nm compared to the control (figure 2(b)). By making use of the multivalency of STV, we also conjugated two biotins on strand B (figure 2(c) inset). This leads to even lower Tm of BS after bivalent binding to STV, which then pushed the generation of more AS. As expected, the assay
now underwent an even more significant color change than the control. The color change in this assay is so pronounced that the difference to the control can be observed by the naked eye directly (figure 2(c)).

It is worth noting that this system can be altered to differentiate single nucleotide polymorphism (supporting information figure S3).

**Colorimetric detection of STV by a one-strand SDC assay with a split G-quadruplex**

All the previous SDC assays include three oligonucleotide strands, which can reach a thermodynamic equilibrium in a 1:1:1 ratio. The initial stoichiometry of the three strands is important, since it has effect on the signal change [9]. One way of perfectly controlling stoichiometry is incorporating all the strands into one long strand [24]. Here we use this strategy to develop a one-strand SDC assay for STV detection. To minimize the number of modifications and still achieve colorimetric sensing, split G-quadruplex peroxidase was employed for readout.

From the 5’ end to 3’ end, the biotin-modified long strand (Lb) as SDC assay is composed of the following regions: one tract of guanines, strand A including toehold 1 (denoted as a) and branch migration region (denoted as b), strand B including a second branch migration region label with a biotin (denoted as another b)
and toehold 2 (denoted as c), a polyT loop as spacer (denoted as d), strand S including complements of c, b and a successively (denoted as c′, b′ and a′ respectively), three tracts of guanines (figure 3(a)). As the classic three-strand system, here region A and region B still compete to hybridize with region S, and only the formation of BS will allow efficient G-quadruplex DNAzyme generation (figure 3(a)).

Because toehold 1 (a) is intentionally designed shorter than toehold 2 (c) (2nt versus 4nt), b′ tends to hybridize with the second b at the beginning. In the presence of STV, the second b lost its advantage to the first b in terms of binding with b′, therefore the duplex of a + b instead of b + c dominated, a configuration favouring the reunion of the two moieties of the G-quadruplex (figure 3(a)). As a result, this binding-induced reconfiguration was observed as a detectable color change (figure 3(c)). Absorbance at 420 nm showed about 2-fold increase in absorbance, compared to almost no signal change in the control system without biotin (figure 3(b)).

**Conclusion**

In conclusion, we have modified our SDC-based assay to explore its versatility for target recognition and as signal transducer. Three different model systems were demonstrated in this study: (1) A structural-switching aptamer was used to replace the toehold on one side, for ATP sensing through aptamer binding; (2) a split G-quadruplex DNAzyme was employed to achieve colorimetric detection of STV; (3) all the three oligonucleotides were integrated into one strand while maintaining similar STV detection capability. The experimental results validated all the three designs. With these developed extensions and further potentials for
modularity, we envision the SDC-based assay to find more applications in biosensing of medically relevant targets.

Note
The authors declare competing financial interests: ZZ and KVG have filed a PCT application (WO2014041024) related to this work.

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