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Short communication

A universal and label-free aptasensor for fluorescent detection of ATP and thrombin based on SYBR Green I dye

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ABSTRACT

A facile and universal aptamer-based label-free approach for selective and sensitive fluorescence detection of proteins and small biomolecules by using the SYBR Green I (SGI) dye is developed. This robust versatile biosensing strategy relies on fluorescence turn-off changes of SGI, resulting from target-induced structure switching of aptamers. Upon binding with the targets, the aptamers dissociate from the respective cDNA/aptamer duplexes, leading to the release of the dsDNA-intercalated SGI into solution and the quenching of the corresponding fluorescence intensities. Such target-induced conformational changes and release of aptamers from the DNA duplexes essentially lead to the change in the fluorescence signal of the SGI and thus constitute the mechanism of our aptamer-based label-free fluorescence biosensor for specific target analyses. Under optimized conditions, our method exhibits high sensitivity and selectivity for the quantification of ATP and thrombin with low detection limits (23.4 nM and 1.1 nM, respectively). Compared with previous reported methods for aptamer-based detection of ATP and thrombin, this label-free approach is selective, simple, convenient and cost-efficient without any chemical labeling of the probe or the target. Therefore, the present strategy could be easily applicable to biosensors that target a wide range of biomolecules.

1. Introduction

Aptamers are synthetic oligonucleotides that could be *in vitro* generated by SELEX (systematic evolution of ligands by exponential enrichment) technology (Tuerk and Gold, 1990; Famulok and Szostak, 1992; Osborn and Ellington, 1997). Due to their highly specific binding affinity toward a variety of targets, including small bio-/organic/inorganic molecules (Huizenga and Szostak, 1995; Wang et al., 2005; Sankaran et al., 2006), proteins (Lou et al., 2009), drugs, metal ions and even cell surfaces (Sefah et al., 2010), aptamers have been widely used as recognition elements for biochemical research (Jameson and Ross, 2010), drug delivery (Zhang et al., 2011), molecular imaging (Kim et al., 2010; Yu et al., 2011) and clinical diagnosis (Ye and Yin, 2008). Compared with conventional affinity reagents (e.g., antibodies), aptamer probes possess several advantageous features, such as comparable or better affinity than that of antibodies, easy production, chemical stability and flexibility, in biosensor design. These unique characteristics of aptamers make them the ideal recognition elements in designing different types of electrochemical (Li et al., 2010; Wu et al., 2008), fluorescence (Zheng et al., 2012; Tan et al., 2012) or colorimetric (Zhu et al., 2011; Ho and Leclerc, 2004) sensing schemes for a broad-spectrum of targets.

Fluorescence biosensing is one of the most popular methods for the design of aptamer-based sensing systems, largely due to its advantages such as simple manipulation, high sensitivity and potential for high-throughput bioanalysis. However, most reported aptasensors require dual-label modifications of the apatmer probes with fluorophores and quenchers (Stojanovic et al., 2001, 2000; ; Tang et al., 2008; Kim et al., 2012; Zhang et al., 2010) or fluorophore donors and acceptors (Zhang et al., 2009; Qiu et al., 2010; Hiller et al., 2003; Ma et al., 2007; Urata et al., 2009; Ray et al., 2006; Hasegawa et al., 2008), which is laborious, time-consuming and expensive. Furthermore, dual labeling of aptamers can often weaken the binding affinities between aptamers and the corresponding targets (Choi et al., 2009) and influence the sensitivity for detection. Therefore, much research efforts have been directed to the development of convenient and label-free methods for fluorescence detection of different target molecules. In this regard, a number of label-free aptamer-based sensing methods have been reported by utilizing different fluorescent reporters, including DNA structure selective binding fluorescent dyes (Choi et al., 2009; Li et al., 2010; Qin et al., 2010; Huang and Chang, 2008) and conjugated polymers (Ho and Leclerc, 2004; Lee et al., 2008), to transduce the fluorescence signals as conformational changes of aptamers upon binding with targets. However, one of the limitations of these

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approaches is the requirement of extensive optimization of oligonucleotide sequences in order to control the binding sites between the dyes and DNA, which limits the application of the method for the assay of general targets (Cekan et al., 2009). In addition, most fluorescent reporters mentioned above are not commercially available and complicated synthesis routes are required to obtain these reporters. The lack of general availability of the reporters further limits the applications of those methods. Therefore, it is highly desirable to develop a universal, label-free and simple approach for the facile detection of non-nucleic acid targets with less structure modifications on both the aptamers and the targets.

Here, we report on the development of a universal and label-free aptamer-based fluorescence strategy for the detection of proteins and small biomolecules using commercially available SYBR Green I (SGI) dye as the signal probe based on its high specificity for dsDNA and bright fluorescence upon intercalation into dsDNA. For proof-of-concept demonstration, two biologically important molecules, ATP and thrombin, were selected as the model targets. ATP is the primary energy carrier of the living systems and aberrant ATP levels can be associated with a variety of genetic diseases, while thrombin has been recognized as the biomarker for coagulation and cardiovascular disease therapy (Zhu et al., 2006). Convenient and sensitive detection of these important biomolecules would thus potentially benefit early diagnosis and treatment of different types of diseases. Our approach for ATP and thrombin monitoring relies on target-induced release of aptamers from the cDNA/aptamer duplexes in solution, accompanied by a significant decrease of fluorescence of SGI. Thus, the specific interactions between the target and aptamer can be investigated by monitoring the change of SGI fluorescence intensity. This approach offers great potential for simple, sensitive and cost-effective detection of various aptamer specific binding targets.

2. Experimental

2.1. Materials and reagents

Thrombin, mouse IgG, lysozyme and BSA were purchased from Sigma (St. Louis, MO). ATP, cytidine triphosphate (CTP), uridine triphosphate (UTP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and guanosine triphosphate (GTP) were purchased from Worthington Biochemical (Lakewood, NJ, USA). The thrombin and ATP binding aptamers (noted as TBA and ABA, respectively) as well as the corresponding complementary DNAs (cDNAs) were synthesized and purified by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China) with the following sequences:

TBA: 5′-GTGGTAGGGCAGGTTGGGGTGACT-3′;
cTBA: 5′-GTGTGTAGTCACCCCAACCTGCCC-3′;
ABA: 5′-ACCTGGGGGAGTATTGCGGAGGAAGGT-3′;
cABA: 5′-CTTCCTCCGCAATACTCCCCCAGGT-3′.

SGI (10,000 ×) was purchased from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China). The as received SGI was firstly diluted to 500 × with dimethyl sulfoxide (DMSO) to make a stock solution, which was freshly diluted to a concentration of 19.8 μM based on the Lambert-Beer law (Zipper et al., 2004) with ultrapure water before use. All reagents were of analytical grade and solutions were prepared using ultrapure water (specific resistance of 18 MΩ•cm).

2.2. Label-free aptamer-based sensing protocol

The aptamers (10 μM) were first hybridized with their complementary cDNAs (10 μM) to form dsDNAs (TBA/cTBA and ABA/cABA) by mixing them in the annealing buffer (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.5) and heating at 95 °C for 10 min, followed by slowly cooling down to room temperature. Finally, the mixture was incubated overnight at 4 °C.

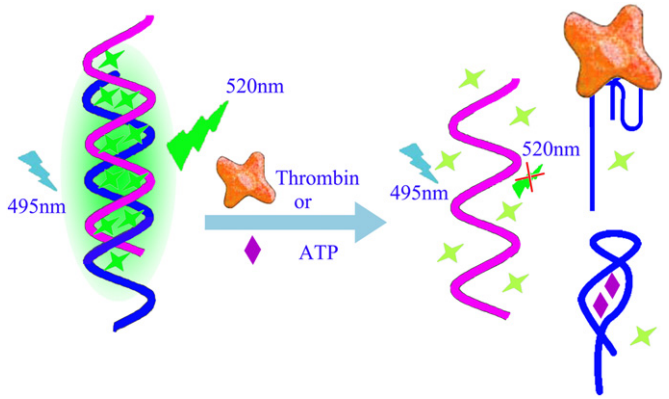
For aptamer/target binding detections, 7.4 μL of SGI (19.6 μM) was added to the solution containing 50 nM aptamer/cDNA and incubated for a suitable time at room temperature. Subsequently various amounts of target were added and incubated for 10 min, immediately followed by fluorescence measurements.

2.3. Fluorescence measurements

Fluorescence spectra data were collected with an RF-5301PC spectrophotometer (Shimadzu, Tokyo, Japan) equipped with a 150 W xenon lamp (Ushio Inc, Japan), excitation at 495 nm and an emission range from 480 to 650 nm. Slit widths for the excitation and emission were set at 3 and 5 nm, respectively. All measurements were performed in 50 mM Tris–HCl containing 100 mM NaCl and 10 mM $MgCl_2$ at pH 7.5.

3. Results and discussion

In this work, we propose an aptamer-based, label-free fluorescence strategy for the detection of proteins and small biomolecules, employing thrombin and ATP as the model target analytes. The sensing mechanism of the proposed method for quantitative detection of thrombin and ATP is illustrated in Scheme 1. In our design, the SGI is an asymmetrical cyanine dye and the fluorescence of free SGI is very weak. However, the fluorescence of SGI can be dramatically enhanced upon binding to dsDNA (with excitation and emission maxima at 495 and 520 nm, respectively) while no significant fluorescence change can be observed with its binding to ssDNA. This unique phenomenon of SGI has laid the foundation for the development of the universal, label-free aptasensor herein. In the absence of the target analyte, the SGI binds to the aptamer/cDNA duplexes and exhibits strong fluorescence. On the contrary, upon the addition of the target, the aptamer associates with the target and releases the cDNA from the dsDNA and reduced fluorescence is expected due to the specific intercalation of SGI to dsDNA rather than ssDNA. Therefore, quantitative analysis of ATP and thrombin can



Scheme 1. Schematic illustration of the universal and label-free fluorescence assay protocol for the detection of ATP and thrombin based on target-induced conformational changes of aptamers using SGI.

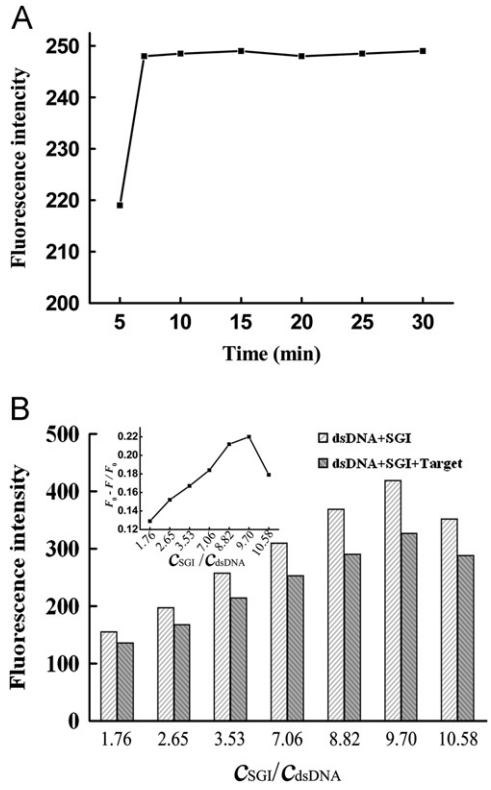


Fig. 1. (A) Effect of the incubation time between SGI (353 nM) and aptamer/cDNA (50 nM) upon the fluorescence intensity in the reaction buffer. (B) Effect of the concentration ratio of SGI to aptamer/cDNA duplex (50 nM) on the fluorescence intensity with and without the presence of 100 μM ATP. Inset: plot of $(F_0-F)/F_0$ versus c_{SGI}/c_{dsDNA}.

be achieved by monitoring the fluorescence intensity change of SGI.

Since our approach relies on the fluorescence change of SGI before and after the addition of the target, it is important to ensure that the decrease of fluorescence is attributed only to the specific interaction between the target and aptamer. We first optimized the intercalation time of SGI to the aptamer/cDNA duplexes. The fluorescence was recorded between 5 and 30 min with a 5 min interval upon incubation of 353 nM SGI with 50 nM aptamer/cDNA (ABA/cABA) duplexes and the results are shown in Fig. 1A. From this figure, we can see that the fluorescence intensity increases rapidly from 5 min to 10 min and levels off thereafter. Therefore, a 10 min pre-incubation of SGI with the aptamer/cDNA duplexes was performed before the introduction of the target into the sensing system.

Considering that our sensing strategy is based on the change of fluorescence of SGI that binds to the aptamer/cDNA duplexes, the concentration of SGI may affect the assay performance. In this regard, the effect of the concentration of SGI on the performance of the assay protocol was evaluated by varying the ratio of SGI to aptamer/cDNA in the presence of 100 μM ATP. The fluorescence responses were studied by measuring the fluorescence quenching efficiency ($[F_0-F]/F_0$), where F_0 and F are the fluorescence intensity of SGI–aptamer/cDNA in the absence and presence of the target, respectively. According to Fig. 1B, the fluorescence intensity of SGI–aptamer/cDNA increases with increasing c_{SGI}/c_{dsDNA} ratio without the presence of the target ATP. The addition of 100 μM ATP at different ratios of c_{SGI}/c_{dsDNA} leads to decreases in fluorescence intensity as discussed previously and the ratio c_{SGI}/c_{dsDNA}= 9.70 exhibits the best fluorescence quenching efficiency (inset). Based on these results, 485 nM SGI and 50 nM dsDNA (c_{SGI}/c_{dsDNA}= 9.70) were used in subsequent experiments.

Toward the demonstration of the universal detection capability of our sensing protocol, we first evaluated the proposed aptamer-based label-free fluorescence strategy for the detection of ATP. As displayed in Fig. 2A, the presence of increasing concentration of ATP from 0 to 10 mM (curves b–h) leads to a gradual decrease of the fluorescence intensity of the intercalated SGI compared with that in the absence of ATP (curve a), which reflects the binding between ATP and the corresponding aptamer and the dissociation of the aptamer from the aptamer/cDNA duplex as well. The linear relationship of the fluorescence signal decrease (F_0–F) vs the target concentration in the inset of Fig. 2A indicates that ATP concentration can be quantitatively derived, and the detection limit (defined as 3σ/slope, where σ is the relative standard deviation of a blank solution, n=11) is calculated to be 23.4 nM. Although such detection limit is about 23-fold higher when compared with the gold standard luminescent firefly luciferase assay method (Invitrogen ATP Determination Kit, detection limit: 1 nM), it is capable of monitoring ATP variations in human plasma, which was reported to be ~1 μM (Lader et al., 2000; Gorman et al., 2007), with greatly reduced cost and time by avoiding using any enzyme.

The specificity of the assay protocol relies on the high selectivity of the aptamer. We investigated the specificity of the aptasensor for ATP against its analogs, including GTP, UTP, CTP, ADP and AMP. As shown in Fig. 2B, the specific target analyte (ATP, 100 μM) leads to a significant decrease in the fluorescence intensity while the control molecules even with 10-fold excess (1 mM) than the target ATP show minimal decreases. These results clearly indicate that the aptamer-based turn-off fluorescence aptasensor is highly specific for ATP determination.

To verify the general applicability of our proposed sensing design, thrombin was also monitored by using this label-free approach. As shown in Fig. 2C, the intensity of the fluorescence decreases accordingly when the concentration of thrombin is increased from 0 nM to 500 nM. The inset in Fig. 2C shows the calibration plot of the fluorescence signal decrease (F_0–F) vs. $\log c_{thrombin}$. A good linear relationship can be obtained under the optimum conditions. The detection limit for thrombin is approximately 1.1 nM. Our method shows better analytical performance for thrombin detection over one of the recent reported similar label-free approach, which was based on exonuclease I and SYBR Gold dye fluorescent indicators (Zheng et al., 2012). Moreover, our approach for thrombin monitoring avoids using enzymes and can be completed in 20 min at room temperature (versus 40 min at 37 °C), which significantly simplify and reduce the cost for the assay method.

The selectivity of this aptamer-based fluorescence turn-off method toward thrombin (100 nM) was also evaluated against three non-target proteins (IgG, lysozyme and BSA at 1 μM). As displayed in Fig. 3D, the presence of excess non-target proteins leads to insignificant changes in fluorescence intensity. These results further confirm the high selectivity of the sensing system.

4. Conclusion

In summary, the present study demonstrated a universal aptamer-based, label-free fluorescence strategy for the detection of proteins and small biomolecules. The assay principle relies on a SGI fluorescence turn-off mechanism based on target-induced release of aptamer from the aptamer/cDNA duplex. Under optimized conditions, this strategy shows high sensitivity and selectivity for the quantitative detection of ATP and thrombin with a low detection limit of 23.4 and 1.1 nM, respectively. The proposed detection scheme herein exhibits major advantages in terms of simplicity and general applicability. In our assay protocol,

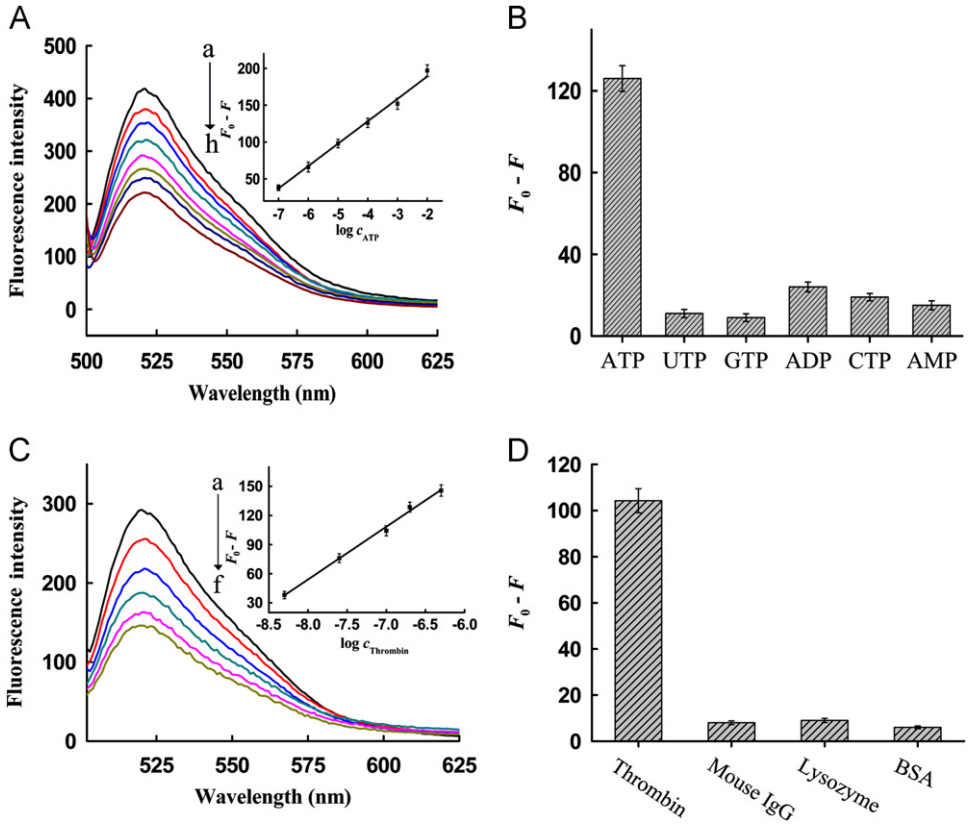


Fig. 2. (A) Fluorescence spectra of a solution containing 485 nM SGI and 50 nM ABA/cABA after incubation with various concentrations of ATP: (a)–(h) 0 nM, 100 nM, 1 μM, 10 μM, 100 μM, 5 mM, and 10 mM. Inset: calibration plot of $F_0 - F$ against the corresponding log c_{ATP}. (B) Selectivity evaluation of the proposed method for ATP (100 μM) against GTP, UTP, ADP, CTP and AMP at 1 mM. (C) Fluorescence spectra of a solution containing 485 nM SGI and 50 nM TBA/cTBA after incubation with various concentrations of thrombin: (a–i) 0, 5, 25, 100, 200, and 500 nM. Inset: calibration plot of $F_0 - F$ against the corresponding log $c_{thrombin}$ (D) Selectivity evaluation the proposed method for thrombin (100 nM) against mouse IgG, lysozyme and BSA at 1 μM. Error bars: SD, $n = 3$.

expensive aptamer labeling process is avoided, which can protect its binding affinity and significantly reduce the detection cost. Although we showed the monitoring of only ATP and thrombin with the proposed method here, this detection scheme can be expanded to detect other important molecules such as cocaine, lysozyme, PDGF etc. with careful design of the corresponding aptamer/cDNA duplexes.

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