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Enzymatic cleavage and mass amplification strategy for small molecule detection using aptamer-based fluorescence polarization biosensor

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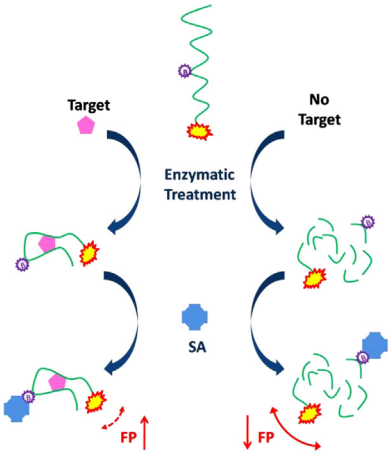
HIGHLIGHTS

- A novel mass amplification strategy was developed.
- This strategy can be used for sensitive detection of small molecule in complex biological samples.
- Excellent performances in adenosine measurement with aptamer-based FP probe.

GRAPHICAL ABSTRACT



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ABSTRACT

Fluorescence polarization (FP) assays incorporated with fluorophore-labeled aptamers have attracted great interest in recent years. However, detecting small molecules through the use of FP assays still remains a challenge because small-molecule binding only results in negligible changes in the molecular weight of the fluorophore-labeled aptamer. To address this issue, we herein report a fluorescence polarization (FP) aptamer assay that incorporates a novel signal amplification strategy for highly sensitive detection of small molecules. In the absence of adenosine, our model target, free FAM-labeled aptamer can be digested by nuclease, resulting in the release of FAM-labeled nucleotide segments from the ***dT***-biotin/streptavidin complex with weak background signal. However, in the presence of target, the FAM-labeled aptamer–target complex protects the FAM-labeled aptamer from nuclease cleavage, allowing streptavidin to act as a molar mass amplifier. The resulting increase in molecular mass and FP intensity of the aptamer–target complex provides improved sensitivity for concentration measurement. The probe could detect adenosine from 0.5 μM to 1000 μM, with a detection limit of 500 nM, showing that the sensitivity of the probe is superior to aptamer-based FP approaches previously reported for adenosine. Importantly, FP could resist environmental interferences, making it useful for complex

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biological samples without any tedious sample pretreatments. Our results demonstrate that this dual-amplified, aptamer-based strategy can be used to design fluorescence polarization probes for rapid, sensitive, and selective measurement of small molecules in complicated biological environment.

1. Introduction

Fluorescence polarization (FP) is a promising choice for fluorescence sensing based on fast, accurate and sensitive signal reporting [1–3]. Moreover, as a ratiometric approach, FP is closely related to its rotational relaxation time, which, in turn, depends on its molecular volume (molecular weight) under constant temperature and solution viscosity [4,5]. Furthermore, FP is insensitive to fluorescence fluctuation and photobleaching, and it can be directly used in complex biological environment. During the past few decades, FP assays have been successfully employed in many research areas, such as protein–protein interaction, protein–DNA interaction and immunoassays for drug discovery, diagnosis, food analysis, and environmental monitoring [6–8].

The emergence of aptamer probes has broadened the use of FP assays in many research areas. Typically, the binding of dye-labeled aptamer to ligand proteins, or any other macromolecules, results in large mass change from free aptamer to aptamer–target complex, allowing FP probes to successfully sense macromolecule dynamics, including, for example, the real-time monitoring of oncogenic protein platelet-derived growth factor [9]. Furthermore, aptamer-based FP has also been widely used for detecting various targets, such as proteins and cancer cells [10–12]. To construct an effective FP sensor, the target molecule should bring change of molecular mass from free probe to target probe. In this sense, binding with a small target would only generate negligible change in molecular weight and global rotation between free fluorophore-labeled probe and fluorophore-labeled probe–target complex, making FP unsuitable for the detection of small molecules.

However, with elegant design based on the unique conformational flexibility of aptamers, some aptamer-based FP probes for small-molecule detection have recently been proposed using competitive displacement, induced-fit binding, and mass amplification strategies [13–16]. Among them, mass amplification using proteins or nanomaterials as amplification moieties has been widely developed based on simple design and effective signal output. For example, Zhu et al. [17] and Cui et al. [18] presented some simple methods to transduce aptamer/small-molecule binding into a detectable signal based on fluorescence anisotropy, as well as allosteric probes, using thrombin or single-stranded DNA binding (SSB) protein as the mass amplifier, respectively. Nonetheless, by their facile deactivation, most proteins are hard to detect and thus unsuitable for practical applications. To address this problem, our group reported a fluorescence anisotropy (FA) signal amplification strategy to sensitively detect small molecules in real time by employing graphene oxide as an amplifier [19]. In addition to mass amplification strategies, the unique induced-fit mechanism of aptamers has also been proposed as an alternative scheme to develop FP biosensors for small-molecule detection [20]. For example, Kidd et al. introduced a method of target-triggered enzymatic cleavage protection in which the absence of target results in the digestion of the aptamer by nuclease, while the presence of target protects the aptamer from digestion by nuclease. Such intact aptamer then allows for relatively higher FP intensity compared to that of cleaved aptamer [21]. All of these methods combine aptamer/target binding with an amplification moiety, making small-molecule detection achievable with FA assays.

Phosphodiesterase I (PDE I) is an exonuclease that successively hydrolyzes 5′-nucleotides from the 3′-hydroxy-termini of both single-stranded and double-stranded DNAs. In our design, PDE I enzymatically cleaved the DNA aptamer substrate in the absence of target molecule, and then released mononucleotides/short single-stranded DNA fragments. The enzymatic cleavage was predicted to be impeded by binding of specific analyte to the functional DNA, resulting in the protection of the aptamer structure. Therefore, target-triggered enzymatic cleavage protection has been combined with streptavidin, as a molar mass amplifier, to produce a dual-amplification strategy that sensitively detects small molecules in homogeneous solution using the FP assay. In the absence of target, nuclease freely and efficiently digests the unbound aptamer into small DNA segments, including dye-labeled DNA segments and biotin-labeled DNA segments, resulting low FP signal of the dye-labeled small DNA segments. However, in the presence of target, the aptamer/target binding complex prevents aptamer cleavage, by conformational or structural changes or by steric hindrance [22,23]. Next, after binding of biotin to the mass amplifier, streptavidin, FP intensity of the aptamer–target complex significantly increases, and dual-amplified detection of small molecules is achieved. As a proof-of-concept, we chose adenosine as the model molecule to demonstrate the feasibility of target-triggered enzymatic cleavage protection and DNA–protein mass amplification, and a detection limit (LOD) of 500 nM was achieved. More importantly, this FP assay was applied to directly detect 3 μM of adenosine in complex biological samples based on its ability to resist environmental interferences. Therefore, this work demonstrates a simple and sensitive aptamer-based FP assay for small-molecule detection, which can be achieved by combining aptamer enzymatic cleavage protection with mass amplification.

2. Materials and methods

2.1. Materials and instrumentation

Streptavidin, adenosine, cytidine, uridine, guanosine and PDE I type IV from *Crotalus atrox* (PDE I) were purchased from Sigma–Aldrich Company (Shanghai, China). RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (heat inactivated, GIBCO) was used as the cell medium. All solutions were prepared in Milli-Q water (resistance >18 MΩ cm) from a Millipore system. Fluorescence measurements were performed on a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, France) with L-format configuration at room temperature. Excitation and emission monochromators were set at 488 nm and 520 nm with 5 nm bandwidth, respectively. All the DNA oligonucleotides were synthesized and purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequence of aptamer-adenosine (Apt-A) was 5′-AG***dT*** GAA CCT GGG GGA GTA TTG CGG AGG AAG GT-3′ with fluorescein dye at the 5′-end and ***dT*** labeled by biotin. The sequence of aptamer-adenosine-1 (Apt-A-1) was 5′-AGT GAA CCT GGG GGA GTA TTG CGG AGG AAG GT-3′ with fluorescein dye at the 5′-end. Tris-buffer solution (20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM NaCl) was used throughout the experiments.

2.2. Fluorescence measurements

Various PDE I solutions ranging from C_0/500 to C_0/1500 were prepared by dilution of the C_0 solution (2 units mL^{-1}) in Milli-Q

water. In the sensing assay of adenosine, the solutions of target (10 μL) and aptamer (10 μL) were added to 70 μL Tris-buffer solution, followed by incubating the mixture under room temperature for 10 min. After pretreatment, the PDE I solution (10 μL) was added, and the reaction was allowed to proceed at 30 °C for 30 min. To terminate the reaction, urea (4 M, 25 μL) was added to the reaction system, followed by incubation for 10 min. Finally, with the addition of streptavidin (5 μL), the mixture was equilibrated at room temperature for 5 min, followed by measuring the fluorescence polarization (FP) at 30 °C.

2.3. Gel electrophoresis analysis

The 2% agarose gels containing 2 μL ethidium bromide per 50 mL gel were prepared using TBE buffer (1×). A volume of 10 μL of different reaction products with 2 μL of loading buffer (6×) was mixed and added to each lane. Agarose gels ran for 40 min at 120 V and then visualized under UV light.

2.4. Parameter determination [5]

The fluorescence polarization value P (mP; 1 P = 1000 mP) is sensitive to changes in the rotational motion of fluorophores, which can be calculated by the Perrin equation

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho}\right) \quad (1)$$

$$\rho=\frac{3\eta Mr(v+h)}{RT}, \quad (2)$$

where τ is the fluorescence lifetime, h is the viscosity of the solution, T is the temperature in Kelvin, R is the gas constant, v is the volume of the rotating unit, η is the viscosity in poise, and P_0 is the limiting polarization. The P value of a fluorophore is proportional to the viscosity of the solvent (η), size (v), and its rotational relaxation time, which, in turn, depends upon its molecular volume (molecular weight).

3. Results and discussion

3.1. Rational design of aptamer-based FP biosensor

The working principle of the designed aptamer-based FP probe is depicted in Fig. 1. In detail, the probe is designed on the basis of target-triggered enzymatic cleavage protection and the extraordinarily strong binding affinity between biotin and streptavidin, a high molecular mass molecule that acts as a polarization amplifier. The aptamer was labeled with a FAM dye at the 5′-end, and a ***dT***-biotin was inserted in the middle of the aptamer. The insertion of biotin did not affect the target binding affinity of the aptamer, as shown in Fig. S1. In the absence of adenosine, the single-stranded Apt-A was vulnerable to PDE I, resulting in the release of cleaved FAM-labeled nucleotide segments, free nucleotide segments and biotin labeled ***dT*** nucleotide segment, respectively. On the other hand, the enzymatic cleavage was predicted to be impeded by binding of adenosine to the functional DNA of Apt-A, resulting in the protection of the aptamer structure. Therefore, the target-induced cleavage protection of the aptamer could be converted into a visual FP signal. The further introduction of streptavidin resulted in the formation of a DNA–protein complex through specific biotin–streptavidin interaction, leading to a secondary amplification of FP in the presence of target adenosine. It should be noted that, in the absence of adenosine, FAM-labeled nucleotide segments and biotin labeled ***dT*** nucleotide segment were separated. Thus, the addition of streptavidin did not affect FAM-labeled nucleotide segment, leading to a low FP signal.

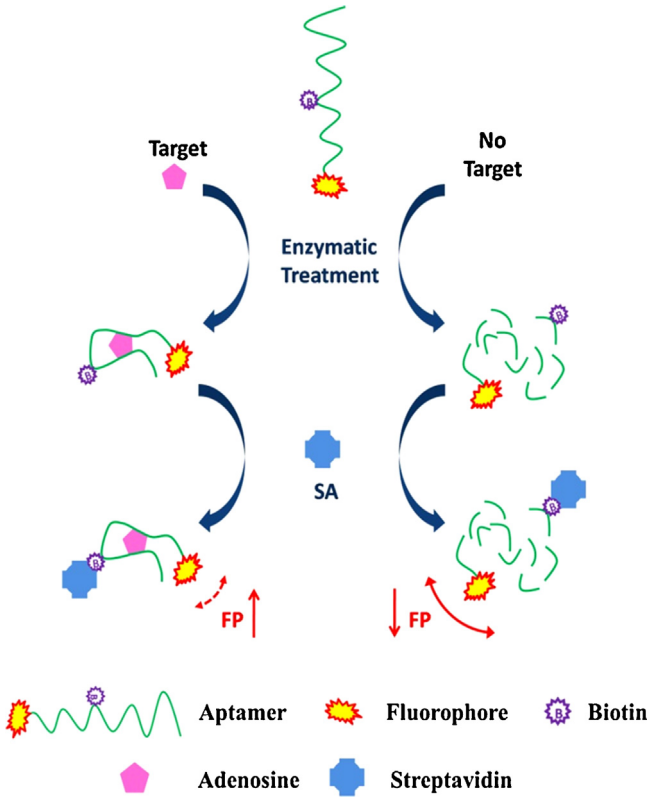

Fig. 1. Scheme of aptamer-based FP sensing platform combined with aptamer enzymatic cleavage protection and mass amplification for adenosine measurement.

3.2. Feasibility of the FP sensing platform

To demonstrate the feasibility of our design, the adenosine aptamer, which is a 32-mer sequence with a molecular mass of 8486, was introduced. Because the molecular mass of adenosine is 267, the binding of anti-adenosine aptamer to adenosine only brings negligible difference in molecular weight between the binding complex and the free aptamer and, hence, no clear change in FP intensity. As shown in Fig. 3, line c, even 1 mM adenosine showed no significant change in FP value.

As noted above, Peyrin et al. reported an alternative strategy termed aptamer enzymatic cleavage protection. In this strategy, by binding of adenosine to the functional DNA of Apt-A, the enzymatic cleavage was predicted to be impeded, resulting in an increased FP response with higher average molecular volume of the weakly cleaved probes. As shown in Fig. 2, gel electrophoresis was first used to confirm the proposed mechanism of this strategy, as applied in the present work. To accomplish this, different concentrations of adenosine were added, and the results showed the appearance of different smeared bands of high molecular weight, which, in turn, served as experimental evidence that the response of the sensing system occurred through adenosine-triggered enzymatic cleavage protection and the extraordinary mass amplification provided by streptavidin while binding to the labeled biotin on the designed aptamer.

The result of aptamer enzymatic cleavage protection triggered by different concentrations of adenosine (5–1000 μM) was then converted into a visual signal (Fig. 3, line b), which varied from 0.0451 to 0.0719 (ΔFP/mP = 26.80). After further addition of

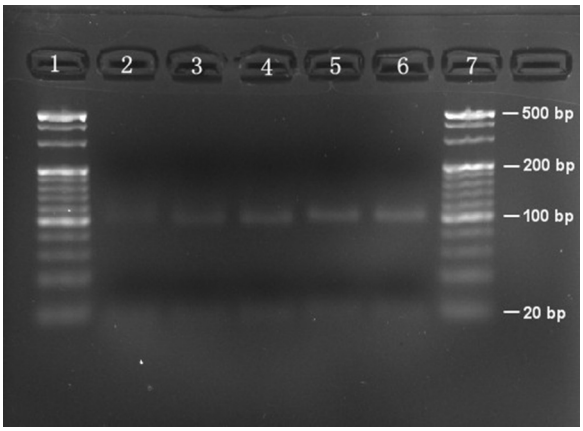

Fig. 2. Agarose gel electrophoresis of FP assay triggered by different concentrations of adenosine: lanes 1 and 7, DNA markers with 20-bp increments; lanes from 2 to 6: 0, 10, 50, 100 and 500 μM adenosine.

streptavidin, a more significant change of FP can be seen in Fig. 3, line a. The FP value was substantially increased from 0.0656 to 0.1518 (ΔFP/mP = 86.20) after introducing 1000 μM adenosine. Combining target-triggered enzymatic cleavage protection with the introduction of streptavidin as a mass amplifier, our strategy could achieve a much lower LOD for small molecules based on FP assay than that achieved by previous methods.

3.3. In vitro *adenosine measurement*

After demonstrating the feasibility of our dual-amplification method, we next verified whether change in FP intensity could be used to sensitively quantify the concentrations of adenosine. To achieve the best sensing performance, the concentration of enzyme, probe, urea, and streptavidin was optimized, as well as reaction conditions, such as temperature and time. With and without the presence of target adenosine, a remarkable FP signal difference was generated from hydrolysis of phosphodiester bonds catalyzed by PDE I, which is critical for the assay (Fig. S2). Since PDE I is an exonuclease that successively hydrolyzes 5′-nucleotides from the 3′-hydroxy-termini of both single-stranded and double-stranded DNAs, the incubation time, temperature and pH value of the reaction system also need to be optimized. Thus, in order to achieve the best performance, the reaction condition was optimized based on signal-to-background ratio (SBR) with a reaction time of 30 min, reaction temperature of 30 °C (Fig. S3) and pH 7.5 (Fig. S4). Moreover, as shown in Fig. S5, the concentrations of aptamer probe, urea and streptavidin were also optimized at 50 nM, 4 M and 5 μg mL^{-1}, respectively. Finally, FP intensity change of Apt-A was studied with a titration assay. As shown in Fig. 4, the ΔFP value increased upon elevating the concentration of the target. The insert figure showed a good linear relationship between adenosine concentration and ΔFP value (0.5–50 μM). The LOD was found to be 500 nM (R^2 = 0.988) based on a SBR > 3, which was better than previously reported methods and even more sensitive than commonly used aptasensing methodologies based on a simple and homogeneous platform, as illustrated in Table 1. As shown in Fig. S6, using the simple method of aptamer enzymatic cleavage protection, we obtained a LOD of 5 μM and a linearity range up to 50 μM (R^2 = 0.984) for adenosine.

The proposed sensing platform was expected to distinguish adenosine from other nucleosides. The FP responses of the sensing system toward three nucleosides, including cytidine, uridine and guanosine, at a concentration of 1 mM were then investigated. In contrast to negligible FP signal changes from various nucleosides, only adenosine displayed an apparent fluorescence polarization change, thus, demonstrating the specificity of our proposed sensing method (Fig. 5).

3.4. Quantitative detection of adenosine in complex biological samples

As a result of ubiquitous endogenous fluorescence interferences from complex biological milieux, differentiation of fluorescence signals remains a problem [4]. It is challenging to detect biomolecules in cell medium since it contains a large amount of autofluorescence species, such as BSA, FBS, riboflavin, nicotinamide, pyridoxine, tryptophan, as well as porphyrin. However, fluorescence polarization may circumvent this problem *via* a ratiometric approach, and to further evaluate the potential effectiveness of our approach in bioassays, cell medium was employed as a complex of fluids for specific adenosine

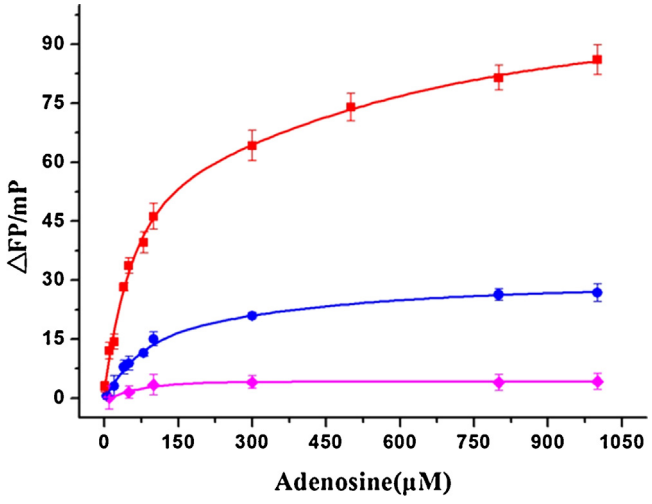

Fig. 3. Plots of fluorescence polarization as a function of adenosine concentration for three modes: target-triggered enzymatic cleavage protection and DNA–protein complex (a, red line), enzymatic treatment, but without streptavidin (b, blue line), and direct assay (c, magenta line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

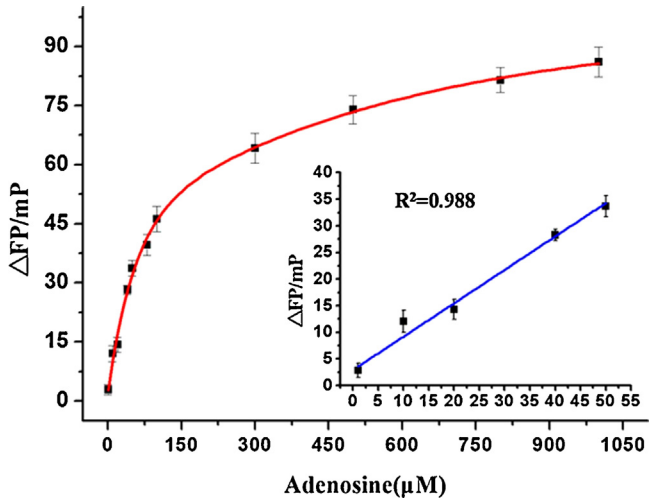

Fig. 4. Fluorescence polarization changes of Apt-A in response to varying concentrations of adenosine. Insert figure: fluorescence polarization changes at low adenosine concentrations.

Table 1
Comparison between the proposed biosensor and other reported biosensors for the detection of adenosine.

Method/technique	Linear range (μmol L^{-1})	Detection limit (μmol L^{-1})	Ref.
Magnetic resonance imaging-based assay	–	500	[24]
Luminescent aptamer biosensor-based assay	60–1000	60	[25]
Enzyme-free and label-free fluorescence aptasensor-based assay	30–680	6	[26]
Aptamer enzymatic cleavage protection-based FP assay	5–25	5	[21]
Aptamer enzymatic cleavage protection and gold nanoparticle-based colorimetric assay	1–100	1	[23]
Aptamer enzymatic cleavage protection and mass amplification-based FP assay	0.5–50	0.5	This work

measurement. In our experiment, the function of Apt-A probe under realistic conditions was examined by spiking biological samples with different concentrations of adenosine (Fig. 6). The result showed that the adenosine titration curves in cell medium (blue line) were similar to those observed in aqueous buffer (red line), with a dynamic range from 5 to 1000 μM. Furthermore, the LOD in cell media was ~1 μM based on 3 times standard deviation of six measurements of blank samples. All these results confirmed that the proposed assay could be practically applied for the direct quantification of adenosine in complex biological samples.

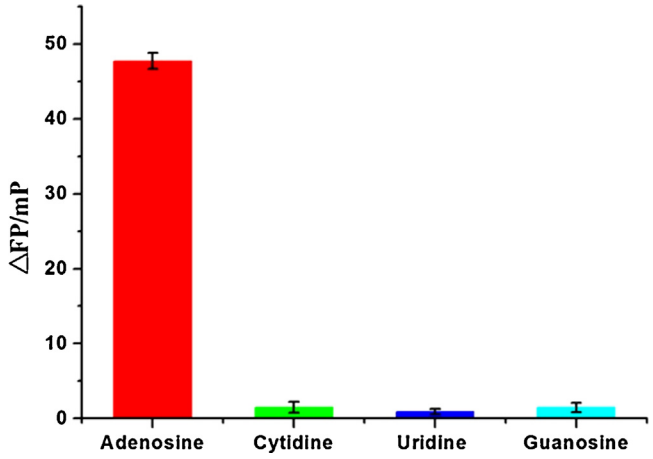

Fig. 5. Selectivity of fluorescence polarization (FP) assay of adenosine over various nucleosides, including cytidine, uridine and guanosine (1 mM).

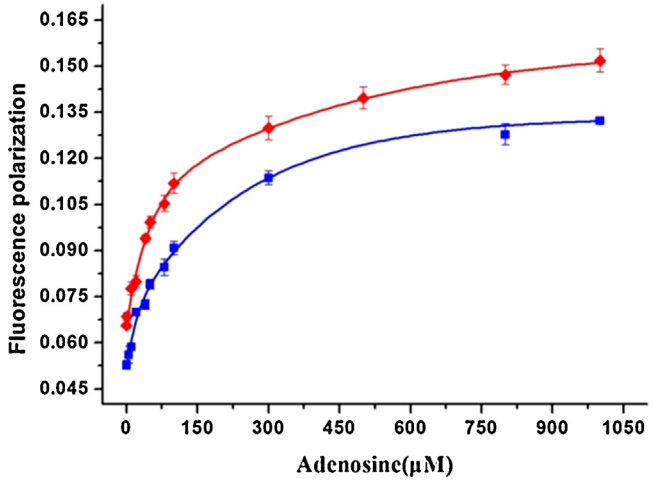

Fig. 6. Plots of fluorescence polarization as a function of adenosine concentration in cell medium (blue line); the response in aqueous buffer is included for comparison (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

In summary, combining target-triggered enzymatic cleavage protection and the extraordinarily strong interaction between biotin and streptavidin, we proposed a novel mass amplification strategy for the sensitive detection of small molecules in homogeneous solution through fluorescence polarization. The proposed assay can be molecularly engineered to offer unique advantages. First, our design makes it possible to use FP as a signal transduction mechanism to construct aptamer probes against small molecules. In contrast to simple target-triggered enzymatic cleavage protection or protein amplification, the combination of target-triggered enzymatic cleavage protection and mass amplification through the use of streptavidin resulted in significant change of mass and, hence, sensitive FP assay with a LOD of 500 nM for adenosine. Second, this strategy is universal. That is, by simply replacing the aptamer with other aptamer sequences, the detection of various targets can be accomplished, such as tyrosine and cocaine. Finally, fluorescence anisotropy can reduce some drawbacks of fluorescence intensity-based assays, such as bleaching and nonuniform emission of the fluorophore, thus, resisting environmental interference and making it possible for direct use in complex biological samples without any tedious sample pretreatment. The unique properties of this proposed strategy will enable the development of a new class of probes for rapid, sensitive, and selective detection of small molecules by means of FP in complex biological samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.03.030.

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