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SHORT COMMUNICATION

Fluorescence polarization biosensor based on an aptamer enzymatic cleavage protection strategy

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Abstract A novel fluorescence polarization (FP) aptasensing platform based on target-induced aptamer enzymatic cleavage protection is reported. The method relies on the FP analysis of the phosphodiesterase I mediated size variation of a dye-labeled aptamer. The tyrosinamide/antityrosinamide DNA aptamer couple was firstly tested as a model system to establish the proof-of-concept. In the absence of the target, the labeled aptamer was enzymatically cleaved into small DNA fragments, leading to a low FP signal. Upon tyrosinamide binding, the DNA substrate was partially protected against the enzymatic attack, leading to an increase in the fluorescence anisotropy response as a result of the higher average molecular volume of the weakly digested probe. The method was subsequently applied to two other systems, i.e., for the detection of ochratoxin A and adenosine. Such an approach was found to combine simplicity and general applicability features.

Keywords Fluorescence polarization · Aptamer · Enzymatic cleavage protection · Small analyte · Sensing

Introduction

Owing to the unprecedented advantages of nucleic acid aptamers as molecular recognition tools, a very great number of aptamer-based assays have been reported using optical [1–3], electrochemical [4–6], mass-based [7], or separation [8–10] methods. Among the optical transduction techniques, the fluorescence-based aptasensing approaches are particularly popular as a result of their intrinsic sensitivity, the possibility to control accurately the labeling location, the availability of many different fluorophores, and the capability for homogeneous, high-throughput, and multiplexed analysis. Several technologies have been successfully used, based on direct, fluorescence resonance energy transfer, fluorescence quenching, light-switching excimer, or fluorescence polarization (FP) methods. The latter constitutes a powerful signal transduction method owing to the requirement of only one labeling reaction, its insensitivity to inner-filter effects, and its ratiometric nature. In this context, numerous FP aptasensors have been described for the detection of both proteins and small analytes during the last few years [11–15]. The aptamer-based FP analysis of macromolecules such as proteins is quite simple, and is related to the molecular volume change of the aptamer probe upon macromolecular target complexation [11, 12]. In contrast, the FP sensing of a small target appears much more complicated as the target binding does not significantly change the size of the labeled functional nucleic acid. To date, two small-target FP aptasensing approaches have been reported, involving direct [13, 14] or displacement [15] mechanisms.

In the direct format, the target binding induces aptamer structural and/or conformational changes that affect precise labeled sites or regions of the functional nucleic acid. These lead to the alteration of the local dye motion, allowing the fluorescence anisotropy signal to be modified [13, 14]. Despite its simplicity and sensitivity, such a strategy is not easily generalizable as it requires a sufficiently large aptamer structural/conformational change [13] or the rational engineering of the aptameric element [14] to produce a detectable fluorescence anisotropy response. In

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the second strategy, the target binding is responsible for the displacement of a dye-labeled complementary strand from the functional nucleic acid, generating a decrease in the fluorescence anisotropy on the basis of the reduction of the apparent size of the probe [15]. Although of wider applicability, such an approach necessitates the tedious case-by-case identification of a suitable complementary strand [1, 16]. Therefore, the development of an alternative FP aptamer-based strategy dedicated to small-molecule sensing would be of great interest.

Previous works have shown that the binding of a target to its specific nucleic acid sequence is able to protect the nucleic acid structure from nuclease cleavage through steric hindrance effects and conformational/structural changes [17, 18]. This feature has been successfully used to study the structural aspects of the complex between targets and their aptamers [19] and to develop sensing methods based on polymerase chain reaction amplification [20]. In the present work, we propose that the nuclease-mediated size variation of a dye-labeled aptamer induced by the target binding can be effectively monitored through the analysis of the fluorescence anisotropy signal change of the fluorescent probe (Fig. 1). In the absence of the target, the labeled aptamer was expected to be enzymatically cleaved into small DNA fragments, leading to a low FP signal. On the other hand, upon target binding, the DNA substrate could be, at least in part, protected against the enzymatic attack. So, an increase in the fluorescence anisotropy response was expected as a result of the higher average molecular volume of the weakly digested probe. Phosphodiesterase I (PDE I), which is able to cleave 5′-nucleotides from the 3′-end of DNA, was employed as a target-responsive enzymatic probe [21]. Two kinds of fluorophores, i.e., Texas red (TR) and fluorescein, were employed as 5′-end labels of the DNA aptamers. The proof-of-concept as well as the general applicability of the FP-based method

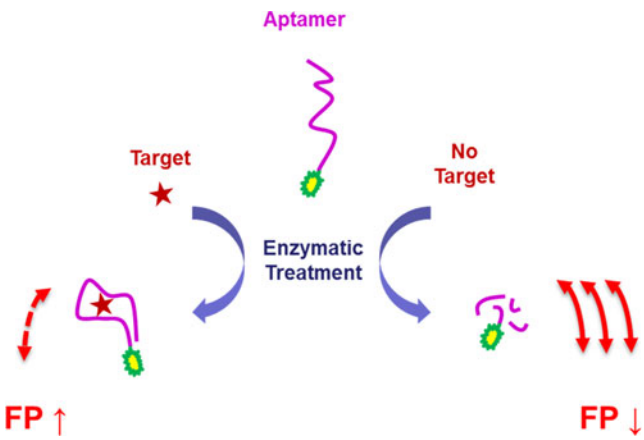

Fig. 1 The aptamer phosphodiesterase I cleavage protection-based fluorescence polarization (*FP*) sensing platform

was investigated using three different aptamer systems corresponding to the following small molecular targets: tyrosinamide (Tym), ochratoxin A (OTA), and adenosine (Ade).

Experimental

Chemicals and apparatus

Tym, tyrosine, phenylalanine, adenosine (Ade), guanosine, inosine, OTA, *N*-acetyltyrosine, urea, tris(hydroxymethyl) aminomethane, and PDE I type IV from *Crotalus atrox* (PDE I) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). NaCl and $MgCl_2$ were purchased from Chimie Plus Laboratoires (Bruyères de Pouilly, France) and Panreac Quimica (Barcelona, Spain), respectively. Water was purified with a Purite Still Plus water system (Thame, UK) fitted with a reverse-osmosis cartridge. Fluorescein- and TR-labeled DNA aptamers were synthesized (the link between the dyes and oligonucleotides was constituted by a C_6 spacer arm) and purified by high-performance liquid chromatography by Eurogentec. The identity of the modified oligonucleotides was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The different aptamer and scramble sequence probes employed were as follows:

Apt-49-T (Tym): 5′-AAT-TCG-CTA-GCT-GGA-GCT-TGG-ATT-GAT-GTG-GTG-TGT-GAG-TGC-GGT-GCC-C-3′ with the 5′-end labeled by TR (Apt-49-TR-T) or fluorescein (Apt-49-F-T); scrambled Apt-49-T: 5′-TGC-ATT-AGA-GCC-GTG-TTT-GGC-GCA-ATT-TGG-CGG-CAG-TTG-TGG-TAG-GTC-G-3′ with the 5′-end labeled by fluorescein.

Apt-36-O (OTA): 5′-GAT-CGG-GTG-TGG-GTG-GCG-TAA-AGG-GAG-CAT-CGG-ACA-3′ with the 5′-end labeled by TR (Apt-36-TR-O) or fluorescein (Apt-36-F-O); scrambled Apt-36-O: 5′-GGC-ATA-GAG-GCG-GCG-AGA-GGT-CTG-TCA-GGT-GTA-GAG-3′ with the 5′-end labeled by fluorescein.

Apt-32-A (Ade): 5′-AGA-GAA-CCT-GGG-GGA-GTA-TTG-CGG-AGG-AAG-GT-3′ with the 5′-end labeled by TR (Apt-32-TR-A) or fluorescein (Apt-32-F-A); scrambled Apt-32-A: 5′-GTG-AAA-GGG-CAA-TGG-CGC-AGT-AGA-GGT-AGG-TG-3′ with the 5′-end labeled by fluorescein.

Fluorescence anisotropy was measured with a Tecan (Männedorf, Switzerland) Infinite F500 microplate reader in black 96-well Greiner Bio-one (Courtaboeuf, France) microplates (reference 675086). The excitation filters used were 485±20 nm for fluorescein and 585±20 nm for TR. The emission filters used were 535±25 nm for fluorescein and 635±30 nm for TR.

Methods

Working solutions

The binding buffer consisted of 20 mM tris(hydroxymethyl)aminomethane–HCl, 20 mM $MgCl_2$, 10 mM NaCl, pH 7.5. Aptamers were prepared in water at a concentration of 2.5 μM and were stored at −20 °C. The working stock solution of the different aptamers at 15.625 nM was prepared daily in 1.25× binding buffer. Before use, it was denaturated at 80 °C for 5 min and left to stand at room temperature for 30 min. The working solution of PDE I was prepared daily by dilution of the C_0 solution (2 units/mL) in deionized water. Various PDE I dilutions ranging from C_0/750 to C_0/2,000 were evaluated as working solutions to find the optimal digestion reaction and assay response. The highest FP signal difference of the aptamer probes, observed in the presence and the absence of the target analyte, was reached for the C_0/1,000 PDE I solution.

Enzymatic treatment

The target (25 μL) was added to 200 μL of the working aptamer solution and the mixture was left to stand for 10 min. Then 25 μL of the C_0/1,000 PDE I solution was added and the reaction proceeded at 25 °C for 45 min. The digestion was stopped by the addition of 62.5 μL of 5 M urea to the 1× binding buffer. After 10 min, the FP was read at 25 °C with a microplate reader with the plates filled with 100 μL of the mixture per well (triplicate experiments). Blank wells were filled with 100 μL of the 1× binding buffer. The fluorescence anisotropy was calculated by the instrument software, as previously described [13, 14]. Calibration curves were plotted by reporting r or $\Delta r = r - r_{min}$ (where r and r_{min} are the anisotropy of the probes submitted to the enzymatic treatment in the presence and absence of the target, respectively) against the target concentration. The different aptamer probes were used at a final concentration of 10 nM.

Results and discussion

To firstly establish the proof-of-concept of the FP sensing platform (Fig. 1), the anti-Tym DNA aptamer (Apt-49-T) was chosen as a model functional nucleic acid (K_d ~2 μM) [13, 22]. Previous experiments on human telomere sequences have demonstrated that the PDE I exonuclease constitutes a useful cleaving probe for application in enzymatic hydrolysis protection assay [21]. As PDE I cleaves nucleotides from the 3′-end of the oligonucleotide, the anti-Tym aptamer was 5′-end-labeled. Two Apt-49-T carrying fluorescein or TR, i.e., Apt-49-TR-T and Apt-49-F-T, were tested as probes. Under the binding buffer conditions used, the maximal value of the fluorescence anisotropy (r_{max}) of the probes (whole, undigested) was approximately 0.170 and approximately 0.090 for Apt-49-TR-T and Apt-49-F-T, respectively. Such a fluorescence anisotropy difference between the two labeled DNA oligonucleotides is consistent with the specific physicochemical properties of the two dyes. In a dye–DNA conjugate, the anionic fluorescein label is known to display a significant degree of local motional freedom, whereas the cationic TR fluorophore typically exhibits a high degree of coupling to the whole DNA [11, 13].

When the two probes were submitted to the optimized enzymatic treatment, in the absence of the target the FP signal decreased strongly. A minimal fluorescence anisotropy value (r_{min}) of approximately 0.085 and approximately 0.035 was obtained for Apt-49-TR-T and Apt-49-F-T, respectively (Fig. 2a). As expected (Fig. 1), the data indicate that the aptamer probes were efficiently digested by the PDE I enzyme, leading to the release of DNA products of smaller size that were characterized by a high level of mobility. The difference between the r_{min} values obtained for the two probes can be attributed to the different physicochemical features of each dye [11].

Upon addition of Tym to the reaction medium before the enzymatic treatment, the FP response of the TR- and fluorescein-labeled aptamer probes was found to be less intensively reduced (Fig. 2a). The Tym/Apt-49-T complex was assumed to resist the enzymatic hydrolysis [19, 21], so the average molecular volume of the probe was higher. The titration curves of the two labeled aptamer probes with increasing Tym concentration were constructed. As shown in Fig. 2a, the addition of the specific analyte from 0 to 1 mM led to an increase in the fluorescence anisotropy from r_{min} to approximately 0.150 and approximately 0.070 for Apt-49-TR-T and Apt-49-F-T, respectively. The relative standard deviations were less than 6% and 3.5% for the TR- and fluorescein-labeled probes, respectively. Over the Tym concentration range studied, the target binding was unable to protect completely the aptameric probes. This was exemplified by the r values obtained at 1 mM concentration which were lower than the r_{max} values. Owing to the high degree of coupling of the TR dye to DNA oligonucleotide, a greater Δr ($r - r_{min}$) value of approximately 0.065 was observed for Apt-49-TR-T at the highest concentration (vs Δr ~0.035 for Apt-49-F-T). In addition, it was previously established that the structural reorganization of an aptamer mediated by the target complexation can affect the fluorescence anisotropy of the nucleic acid probe [13, 14]. For Apt-49-T, under similar experimental binding conditions, the fluorescence anisotropy increase of the probes labeled with TR and fluorescein at the 5′-end in the presence of 1 mM Tym target is 0.007 and 0.005, respectively [13]. Thus, in the present assay, this phenomenon was

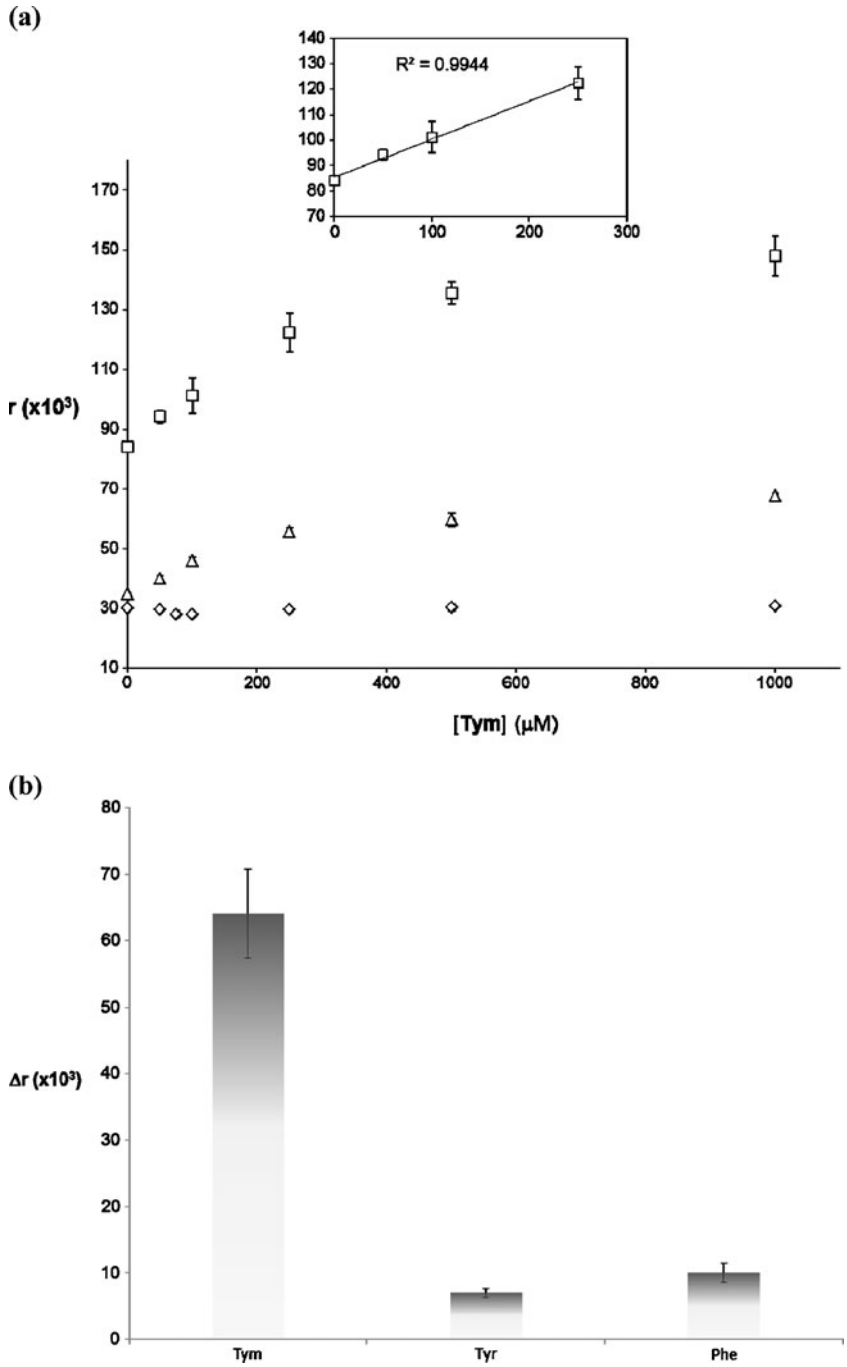

Fig. 2 **a** Calibration curves of aptamer probes with increasing tyrosinamide (*Tym*) concentrations (triplicate experiments): Apt-49-TR-T (*squares*); Apt-49-F-T (*triangles*); scrambled Apt-49-F-T (*diamonds*); see "Chemicals and apparatus" for a description of the probes. The *inset* presents the linear range of the calibration curve obtained using the Apt-49-TR-T probe. **b** Fluorescence anisotropy change $\Delta r = r - r_{min}$ (where r and r_{min} are the anisotropy of the probes submitted to the enzymatic treatment in the presence and absence of the analyte, respectively) for Tym and noncognate ligands (tyrosine, phenylalanine) at a concentration of 1 mM using the Apt-49-TR-T probe

expected to contribute weakly, if at all, to the Δr increase with increasing Tym concentration.

A control experiment was performed using a fluorescein-labeled scrambled sequence 49-mer DNA in place of Apt-49-F-T. The fluorescence anisotropy did not change over the Tym concentration range, demonstrating that the assay response was specifically related to the target binding to the aptameric recognition element (Fig. 2a).

From an analytical performance point of view, a Tym concentration of 15 μM was detected (based on a signal-to-noise ratio above 3) using the Apt-49-TR-T probe with a linear range up to 250 μM (R^2=0.994). The ability of the FP sensing platform to selectively detect Tym was evaluated using Apt-49-TR-T as a probe. Both tyrosine and phenylalanine were tested as noncognate ligands [22]. As shown in Fig. 2b, the two analytes did not cause any significant response over the concentration range, demonstrating the selectivity of the assay.

The general applicability of the strategy was subsequently investigated using two other small target/aptamer systems, i.e., the OTA/anti-OTA aptamer (K_d ~100 nM) [23] and the Ade/anti-Ade aptamer (K_d ~5 μM) [14, 24]. Experiments

were conducted under exactly the same conditions previously used for the anti-Tym aptamer-based assay. The titration curves for the two analytes were constructed by plotting Δr against target concentration, for both TR-labeled and fluorescein-labeled probes in each case (Fig. 3). Identical behavior was obtained for the OTA and Ade aptasensors, consistent with the results reported above for the Tym system. The Δr value increased with increasing target

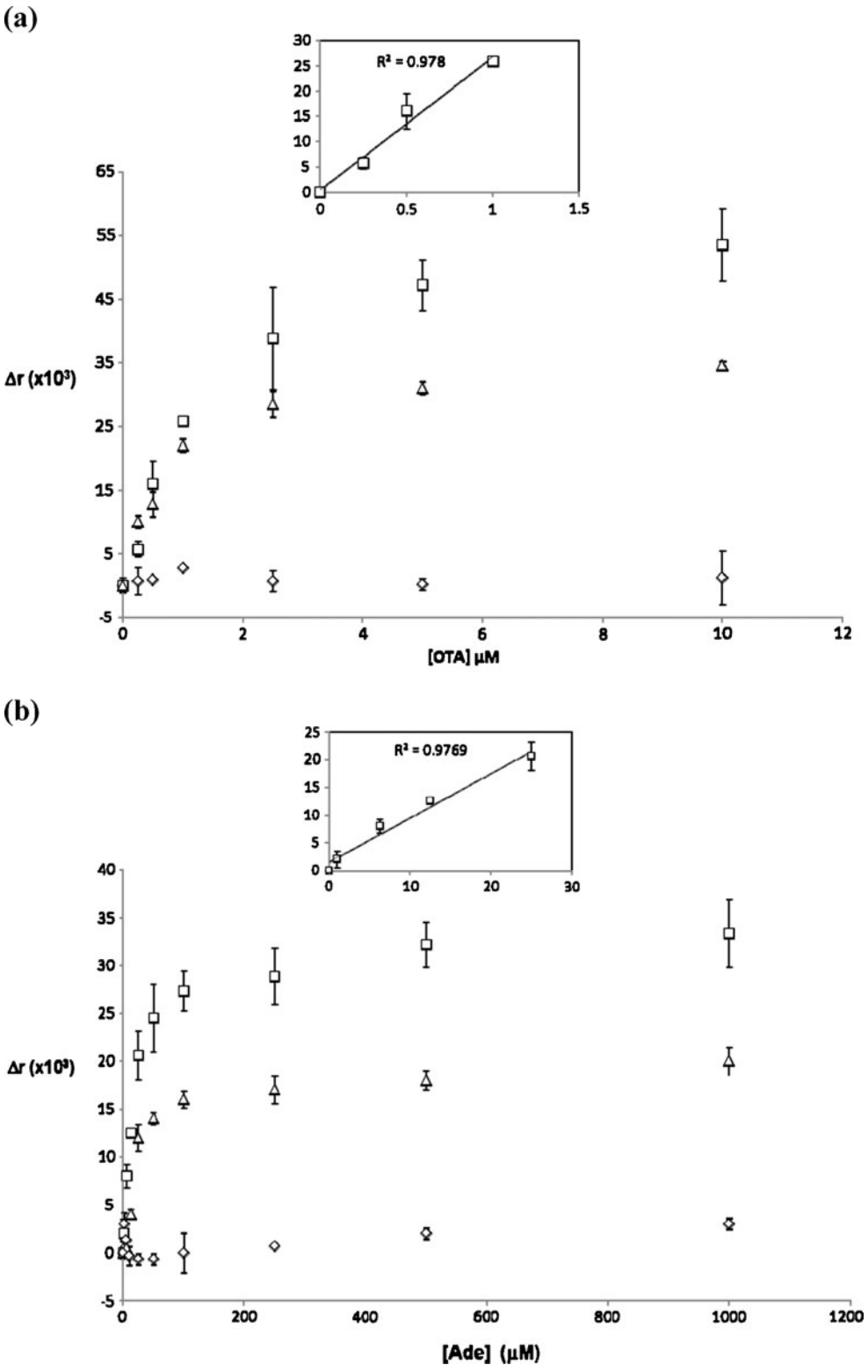

Fig. 3 **a** Calibration curves of aptamer probes with increasing ochratoxin A (*OTA*) concentrations (triplicate experiments): Apt-36-TR-O (*squares*); Apt-36-F-O (*triangles*); scrambled Apt-36-F-O (*diamonds*); see "Chemicals and apparatus" for a description of the probes. **b** Calibration curves of aptamer probes with increasing adenosine (*Ade*) concentrations (triplicate experiments): Apt-32-TR-A (*squares*); Apt-32-F-A (*triangles*); scrambled Apt-32-F-A (*diamonds*); see "Chemicals and apparatus" for a description of the probes. Fluorescence anisotropy change $\Delta r = r - r_{min}$ (where r and r_{min} are the anisotropy of the probes submitted to the enzymatic treatment in the presence and absence of the analyte, respectively). The *insets* present the linear range of the calibration curves obtained using the probes labeled with Texas red

concentration, in accordance with a target-induced protection of the aptamer probe against the PDE I cleavage. A different magnitude of the Δr value in relation to the dye used was still found. Control experiments using scrambled oligonucleotides revealed that the fluorescence anisotropy signal was unchanged in the presence of their respective targets (Fig. 3). Using TR-labeled probes, we obtained a limit of detection of 70 nM and 5 μM and a linearity range up to 1 μM (R^2=0.978) and 25 μM (R^2=0.977) for OTA and Ade, respectively. The cross-reactivity was assessed using *N*-acetyltyrosine [15] and inosine/guanosine [24] for the OTA and Ade systems, respectively. No change in fluorescence anisotropy was obtained over the entire analyte concentration range (data not shown), demonstrating the selectivity the FP approach.

Conclusion

In this short communication, we established the proof-of-concept of a novel FP sensing platform that is based on the aptamer enzymatic cleavage protection strategy. This FP aptasensing approach has at least two interesting characteristics: the ability to function with different and unmodified aptamers and the possibility to work under the same experimental protocol, without the requirement of a tedious case-to-case adaptation. These features, combined with the intrinsic advantages of the fluorescence anisotropy technique, allow us to envisage expanding the potential applications of fluorescent-based aptasensors. It is assumed that such a format will also be applicable to the detection of proteins for which the direct FP method is not optimal, for example, when close contacts between the protein and the dye could result in a weak FP signal response [25].

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