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ORIGINAL PAPER

Signal-amplification detection of small molecules by use of Mg^{2+}- dependent DNAzyme

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Abstract Because small molecules can be beneficial or toxic in biology and the environment, specific and sensitive detection of small molecules is one of the most important objectives of the scientific community. In this study, new signal amplification assays for detection of small molecules based on Mg^{2+}-dependent DNAzyme were developed. A cleavable DNA substrate containing a ribonucleotide, the ends of which were labeled with black hole quencher (BHQ) and 6-carboxyfluorescein (FAM), was used for fluorescence detection. When the small molecule of interest is added to the assay solution, the Mg^{2+}-dependent DNAzyme is activated, facilitating hybridization between the Mg^{2+}-dependent DNAzyme and the DNA substrate. Binding of the substrate to the DNAzyme structure results in hydrolytic cleavage of the substrate in the presence of Mg^{2+} ions. The fluorescence signal was amplified by continuous cleavage of the enzyme substrate. Ochratoxin A (OTA) and adenosine triphosphate (ATP) were used as model analytes in these experiments. This method can detect OTA specifically with a detection limit as low as 140 $pmolL^{-1}$ and detect ATP specifically with a detection limit as low as 13 $nmolL^{-1}$. Moreover, this method is potentially extendable to detection of other small molecules which are able to dissociate the aptamer from the DNAzyme, leading to activation of the DNAzyme.

Keywords Signal amplification · Small molecule · Ochratoxin A · ATP · DNAzyme

Introduction

Small molecules can be beneficial or toxic in biology and the environment, so methods for specific and sensitive detection of small molecules are necessary in many biotechnological applications and in medical diagnostics [1, 2]. Therefore, sensors that can detect and quantify small molecules have been developed for a variety of applications [3–5]. The signal-amplification detection of small molecules has spurred substantial research effort, and numerous electrical, optical, or microgravimetric amplified small-molecule sensors have been reported [6–9]. Such sensors have the advantages that no complicated instrument or sample pretreatment are needed. These amplification approaches include conjugation with enzymes [10], use of catalytic nanoparticles [11], or use of molecular catalysts for DNA recognition complexes [12]. Amplified small molecule detection has also been accomplished by use of an autocatalytic and catabolic DNAzyme-mediated process [13, 14]. Catalytic nucleic acids (DNAzymes or ribozymes) have attracted increasing interest as amplifying labels for biosensing [14–17]. The successful selection of catalytic nucleic acids by use of the systematic evolution of the ligand by exponential enrichment (SELEX) process has led to the use of these biocatalysts as amplifying labels in a variety of biosensing designs [16, 18]. The easy synthetic preparation of DNAzymes, the flexibility in mastering DNAzyme structures by encoding recognition functions into DNAzyme sequences, and the reduced nonspecific absorption of these

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nucleic acids turn the DNAzymes into ideal candidates for the development of bioanalytical devices [19]. Indeed, numerous recent studies have used DNAzymes as amplifying units for bio-sensing [10, 17, 20]. For example, the horseradish peroxidase-mimicking DNAzyme has been extensively used to amplify DNA detection [21, 22] and for the specific amplified sensing of ions [19].

In this paper, we report the protein-free and autocatalytic detection of small molecules by using the Mg^{2+}-dependent DNAzyme as a biocatalyst. At the wavelength of excitation of 6-carboxyfluorescein (FAM), the fluorescence of the FAM label at one end of the DNA substrate was highly quenched by black hole quencher (BHQ) attached at the other end. In the presence of the target molecule, the activated DNAzyme can continuously cleave the DNA substrate, leading to recovery of the fluorescence of FAM. Therefore, the target molecule can be detected and the concentration can be quantified by monitoring the fluorescence intensity of FAM. This method, which we introduce herein, is highly specific and only sensitive to the target molecule. The detection limits for ochratoxin A (OTA) and adenosine triphosphate (ATP) on the basis of use of the corresponding system are 140 $pmol L^{-1}$ and 13 $nmol L^{-1}$, respectively.

Experimental section

Chemicals and materials

Oligonucleotides designed in this study were synthesized by Sangon Biotech (Shanghai, China); the sequences of all oligonucleotides are listed in Table 1. The ribonucleotide-containing DNA substrates used were modified at the 3′ and 5′ ends with the fluorescent dye 6-carboxyfluorescein (6-FAM) and its quencher black hole quencher I (BHQ I), respectively. Other chemicals were of reagent grade and were used without further purification. Solutions were prepared in deionized water processed with a Milli-Q ultra-high-purity-water system (Millipore, Bedford, MA, USA).

Instrumentation

A Cary 500 Scan UV–visible spectrophotometer (Varian, USA) was used to quantify the oligonucleotides. Fluorescence intensities were recorded on a Fluoromax-4 spectrofluorimeter (Horiba Jobin Yvon, France). Emission spectra were recorded in the wavelength range 500–640 nm upon excitation at 492 nm. The slit widths for excitation and emission were set at 2.5 and 15 nm, respectively. All measurements were performed at room temperature unless stated otherwise.

Fluorescence detection of OTA

DNA stock solution was prepared by dissolving oligonucleotides in OTA binding buffer (10 $mmol L^{-1}$ Tris-HCl, 120 $mmol L^{-1}$ NaCl, 5 $mmol L^{-1}$ KCl, 20 $mmol L^{-1}$ $CaCl_2$, and 20 $mmol L^{-1}$ $MgCl_2$, pH8.5) and was stored at 4 °C before use. OTA aptamer (DNA 1) solution (100 μL) of concentration 250 $nmol L^{-1}$ was mixed, in tubes, with 100 μL OTA binding buffer containing different concentrations of OTA, followed by incubation at room temperature for 30 min. Then, 100 μL 250 $nmol L^{-1}$ H_1 (DNA 2) solution and 100 μL 250 $nmol L^{-1}$ H_2 (DNA 3) solution were added, and left to settle for another 30 min. BQF substrate (1 $\mu mol L^{-1}$, 100 μL) was then added, and the mixture was kept at room temperature in the dark for another 1 h. The fluorescence intensity was then measured.

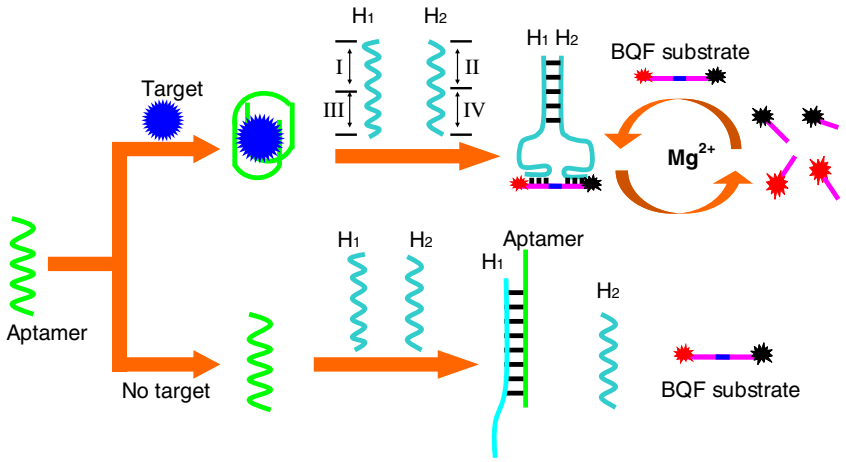
Fluorescence detection of ATP

All assay conditions were the same as those used for the detection of OTA, except that the OTA aptamer (DNA 1) was replaced by ATP aptamer (DNA 4), the H_1 (DNA 2) and H_2 (DNA 3) for OTA were replaced by the H_1 (DNA 5) and H_2 (DNA 6) for ATP, respectively (Table 1), and the OTA binding buffer was replaced by ATP binding buffer (50 $mmol L^{-1}$ Tris-HCl, 140 $mmol L^{-1}$ NaCl, 5 $mmol L^{-1}$ KCl, 20 $mmol L^{-1}$ $MgCl_2$, pH7.5).

Table 1 DNA sequences used in this study

Name	sequence (5′——3′)
DNA 1	5′GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA3′
DNA 2	5′GATATCAGCGATCTTATGTCCGATGCTCCCTT3′
DNA 3	5′ATCGGACAAAGCACCCATGTTACTCT3′
DNA 4	5′ACCTGGGGGAGTATTGCGGAGGAAGGT3′
DNA 5	5′GATATCAGCGATCTTAACCTTCCTCCGCAATA3′
DNA 6	5′GCGGAGGAAGGTAAGCACCCATGTTACTCT3′
BQF substrate	5′Q-AGAGTATrAGGATATC-F3′

F, FAM fluorophore; Q, black hole quencher 1; rA, adenine ribonucleotide



Scheme 1 Illustration of signal-amplification assay for small-molecule detection based on Mg^{2+}-dependent DNAzyme. In the presence of the target, domain I of H_1 hybridizes with domain II of H_2 and forms Mg^{2+}-dependent DNAzyme, which can continuously cleave ribonucleotide-containing DNA substrate, with the result that the FAM moves far away from BHQ, leading to generation of greater fluorescence intensity. In the absence of the target, the aptamer will partly hybridize with H_1, which will prevent formation of the Mg^{2+}-dependent DNAzyme and thus the ribonucleotide-containing DNA is not cleaved

Application

We used red wine and serum samples to confirm the feasibility of this sensor for analysis of real-world samples. Buffer solutions containing 1 % red wine or serum were spiked with different concentration of OTA or ATP and the samples were analyzed as described above.

Results and discussion

Design strategy for detection of small molecules by use of Mg^{2+}-dependent DNAzyme

Scheme 1 illustrates the sensing strategy for detection of small molecules by use of Mg^{2+}-dependent DNAzyme. The DNAzyme is split into two parts, H_1 and H_2. H_1 and H_2 both contain two domains. Domain I in H_1 and domain II in H_2 are complementary DNA sequences which can hybridize into duplexes in the absence of the small molecule's aptamer. Because part of small molecule's aptamer is complementary to domain I in the H_1 sequence, the aptamer can compete with H_2 for hybridization with H_1. Upon addition of the small molecule into the solution containing the aptamer, the aptamer folds into a compact structure and loses the capability to compete with H_2 for hybridization with H_1. Hybridization between H_1 and H_2 brings domain III in H_1 and domain IV in H_2 together to form a functional DNAzyme, which can continuously cleave ribonucleotide-containing DNA substrate [20]. Binding of the substrate to the DNAzyme structure results in hydrolytic cleavage of the substrate in the presence of Mg^{2+} ions [13]. The limited stability of the cleaved substrate and the DNAzyme duplex leads to release of the product units from the DNAzyme structure and enables continuous scission of the substrate [16]. As a result of this scission of the substrate the FAM

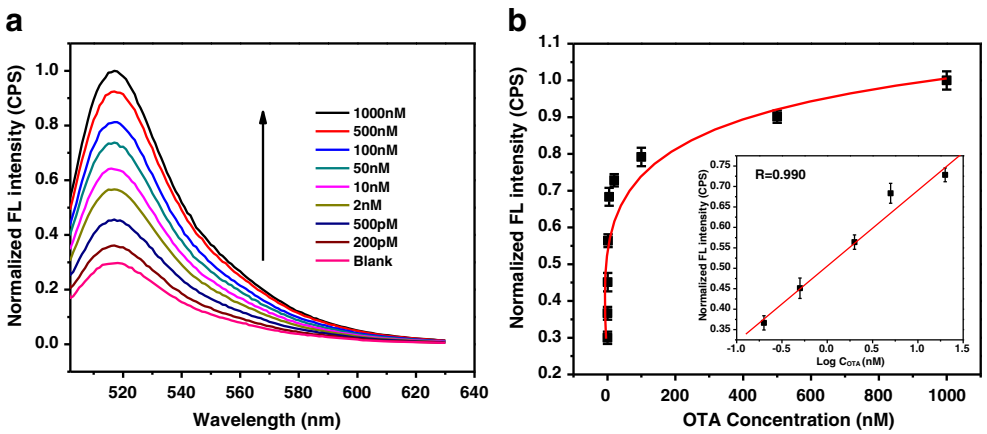


Fig. 1 **a** Fluorescence results for the Mg^{2+}-dependent DNAzyme amplified detection strategy with different concentrations of OTA. **b** The peak maximum of the fluorescence spectra in (**a**) were plotted as a function of concentration of OTA. The *inset* in **b** is the linear part of the plot in **b**. *Error bars* were obtained from three experiments

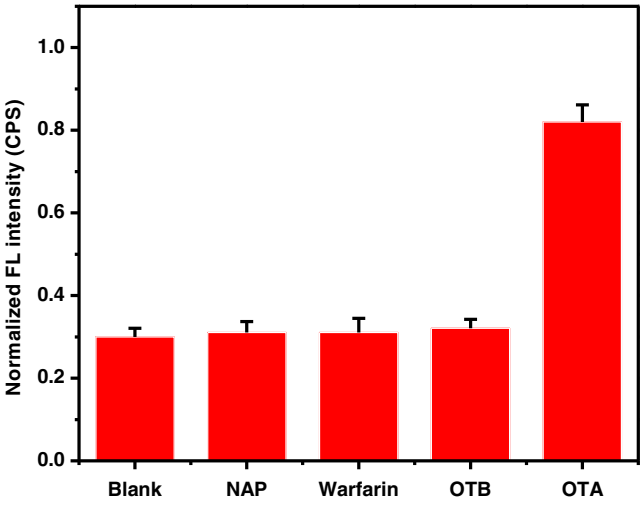


Fig. 2 Selectivity of the sensor for OTA (100 nmolL^{-1}) compared with other structural analogues (1 μmolL^{-1}). *OTA*, ochratoxin A; *OTB*, ochratoxin B; *NAP*, *N*-acetyl-L-phenylalanine. *Error bars* were obtained from three experiments

moves far away from BHQ, leading to generation of greater fluorescence intensity, thus providing the optical readout signal for sensing of the analyte. On the basis of this design, we have developed a signal amplification assay for detection of small molecules. For each small molecule target, the detailed DNA sequence is different whereas the domains in H_1 and H_2 used to form the DNAzyme are fixed.

Analytical performance of OTA detection

To prove the feasibility of the proposed method, we started with detection of ochratoxin A (OTA). OTA, a toxin produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, is one of the most abundant food-contaminating mycotoxins in the world [23, 24]. It has been classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen [25, 26]. With recognition of its severe toxic effect, substantial effort has been devoted to developing simple sensing devices for detection of this fungal toxin [27–30]. In our previous work, we designed a sensing strategy based on single-walled carbon nanotubes and aptamers for detection of OTA [23] without signal amplification. To improve the sensitivity of OTA detection, we introduced this signal-amplification strategy based on Mg^{2+}-dependent DNAzyme. In this signal-amplification strategy, an aptamer specific for OTA (DNA 1) was used. This aptamer can fold into an antiparallel G-quadruplex structure upon exposure to OTA [23]. Formation of this antiparallel G-quadruplex structure prevents hybridization between DNA 1 and domain I of H_1. As a result the supramolecular structure of the DNAzyme (subunits H_1 and H_2) was formed and the substrate was cleaved, producing strong fluorescence.

Premixing of DNA 1 and OTA is important in this study, because it enables sufficient freedom of interaction between aptamer and OTA. As illustrated in Fig. 1a, fluorescence measurement showed that the fluorescence intensity in the solution increased with increasing concentration of OTA. The calibration curve for fluorescence intensity as a function of concentration from 200 pmol L^{-1} to 1000 nmolL^{-1} was plotted (Fig. 1b). The limit of detection (LOD), defined as the concentration corresponding to a fluorescence signal equal to three times the standard deviation of blank without OTA, was calculated to be 140 pmol L^{-1}. The fluorescence response was a linear function of the logarithm of OTA concentration between 200 pmol L^{-1} and 50 nmolL^{-1} (inset in Fig. 1b). Compared with another recently developed sensor for detection of OTA on the basis of a traditional fluorophore quencher [31], this signal-amplification assay greatly enhanced the sensitivity of OTA detection—the LOD was reduced by more than a factor of 14.

To determine the specificity of this method, we tested the method with different structural analogues. *N*-Acetyl-L-phenylalanine (NAP), the molecular structure of which includes one part of OTA, was used. Warfarin and OTA are similar in

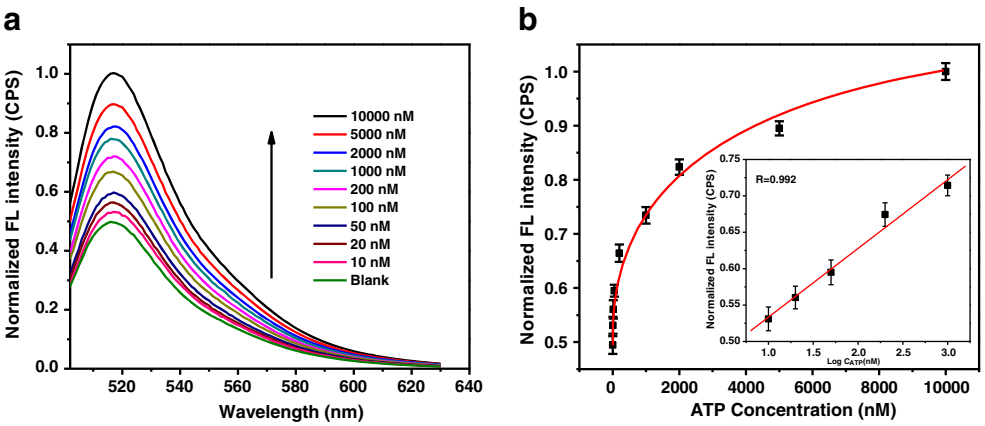


Fig. 3 **a** Fluorescence results for the Mg^{2+}-dependent DNAzyme amplified detection strategy with different concentrations of ATP. **b** The peak maximum of the fluorescence spectra in (**a**) were plotted as a function of concentration of ATP. The *inset* in **b** is the linear part of the plot in **b**. *Error bars* were obtained from three experiments

that that they occupy the same binding site in human serum albumin. Ochratoxin B, which lacks the chlorine atom in the isocoumarin ring is a structural analogue of OTA. As shown in Fig. 2 (column 5), addition of the target molecule (OTA) at a concentration of 100 nmolL^{-1} induced a dramatic increase in fluorescence whereas other analogues at concentrations of 1 μmolL^{-1} (columns 2–4) did not induce apparent fluorescence increase. Therefore, we can conclude that this aptamer was highly specific for OTA.

Analytical performance of ATP detection

To exploit the extendable applicability of our approach, we modified the assay for ATP detection. ATP is the major energy carrier of all living cells and is of crucial importance in the regulation of cellular metabolism and biochemical pathways in every organism [32–34]. As a consequence, ATP concentrations are tightly regulated under normal conditions. Aberrant ATP levels have been associated with particular diseases, for example angiocardiopathy, which results from excessive production of ATP by creatine kinase [35]. Therefore, accurate detection and quantification of ATP is an important objective for both biochemical and clinical applications. In this signal-amplification strategy, an aptamer specific for ATP (DNA 4) was used. This folds to form an antiparallel G-quadruplex structure upon exposure to ATP [35]. Similar to OTA detection, premixing of DNA 4 and ATP was important to enable sufficient freedom of interaction between aptamer and ATP. For the sensitivity study, different concentrations of ATP solution were investigated. Figure 3a shows the fluorescence emission spectra in the presence of ATP from 0.01 to 10 μmolL^{-1} in Tris-HCl buffer solution. With increasing concentration of ATP, the fluorescence intensity was clearly enhanced. Figure 3b shows the relationship between fluorescence intensity and concentration of ATP. The inset shows the calibration curve for quantitative analysis of ATP. The fluorescence response was a linear function of the logarithm of ATP concentration in the range 10 nmolL^{-1} and 2 μmolL^{-1} (inset in Fig. 3b), and the detection limit was 13 nmolL^{-1}. Importantly, as

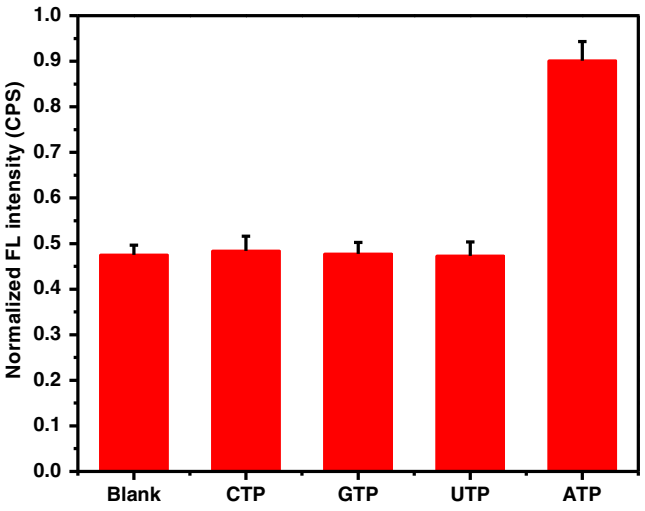


Fig. 4 Selectivity of the sensor for ATP (5 μmolL^{-1}) compared with other structural analogues (50 μmolL^{-1}). *CTP*, cytidine triphosphate; *GTP*, guanosine triphosphate; *UTP*, uridine triphosphate. *Error bars* were obtained from three experiments

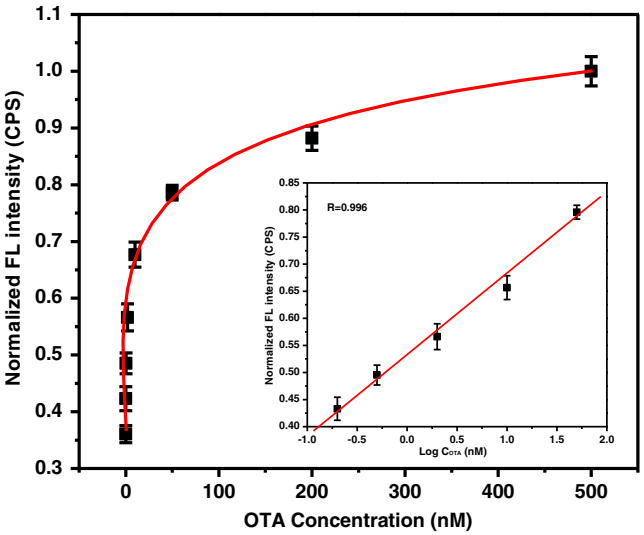


Fig. 5 Peak maxima of the fluorescence spectra of buffer solutions containing 1 % red wine spiked with different concentrations of OTA under the conditions of the amplified detection strategy. The *inset* shows fluorescence response plotted against OTA concentration. *Error bars* were obtained from three experiments

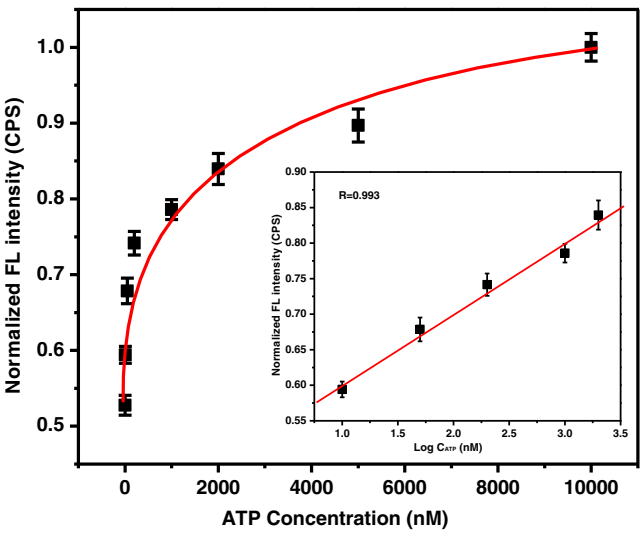


Fig. 6 Peak maxima of the fluorescence spectra of buffer solutions containing 1 % human serum spiked with different concentration of ATP under the conditions of the amplified detection strategy. The *inset* shows fluorescence response plotted against ATP concentration. *Error bars* were obtained from three experiments

shown in Fig. 4, the assay was quite selective. Addition of the target molecule (ATP) at a concentration of 5 $\mu mol L^{-1}$ induced a dramatic fluorescence increase; however, no such fluorescence increase was observed when ATP was replaced by other nucleoside triphosphates, for example CTP, GTP and UTP. Addition of the other nucleoside triphosphates at concentrations of 50 $\mu mol L^{-1}$ (columns 2–4) did not induce any apparent fluorescence increase; therefore, we can conclude that the assay was quite selective.

Practicability of Mg^{2+}-dependent DNAzyme based sensing

To investigate the sensors' potential applications for analysis of real samples, the sensors were used to detect OTA in red wine and ATP in human serum. Calibration curves were obtained for 1 % red wine (Fig. 5) and 1 % serum (Fig. 6) containing different concentrations of analyte (see also Electronic Supplementary Material). Detection limits were 150 $pmol L^{-1}$ for OTA and 14 $nmol L^{-1}$ for ATP, which are close to those obtained in buffer. These results suggest successful detection of OTA and ATP in real samples can be achieved by use of the Mg^{2+}-dependent DNAzyme sensors.

Conclusion

We have developed a protein-free signal-amplification sensing system based on the Mg^{2+}-dependent DNAzyme. The DNAzyme was split into two parts, one of which was partly matched with aptamer to prevent assembly of the catalytically active DNAzyme. Addition of the target molecule enabled hybridization of these two parts and facilitated assembly of the DNAzyme, and the fluorescence signal was amplified by continuous enzyme cleavage. Compared with other sensors [31, 36–38], this DNAzyme system is able to continuously cleave ribonucleotide-containing DNA substrate and the fluorescence intensity is greatly enhanced. In addition to OTA and ATP, this DNAzyme system is universal for other small molecules. It can be foreseen that the simple approach demonstrated here could be modified and coupled with other various detection devices. It might also be useful for high-throughput and paralleled analysis of multiple targets.

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