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A self-assemble aptamer fragment/target complex based high-throughput colorimetric aptasensor using enzyme linked aptamer assay

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ABSTRACT

Enzyme linked aptamer assay (ELAA) uses an aptamer as recognition element and enzyme as signal readout element for establishing different kinds of aptasensors. We reported herein a high-throughput colorimetric aptasensor based on ELAA only requiring a single aptamer sequence for cocaine detection. An anti-cocaine aptamer was cleaved into two fragments, one of which was immobilized on a DNA-BIND 96-well plate via 5′-labeled primary amine and the other one was biotin labeled. The presence of two aptamer fragments and the target molecule led to the formation of aptamer fragments/target complexes. Streptavidin–horseradish peroxidase (SA–HRP) was used to react with biotin in order to obtain quantitative signals. A linear response towards cocaine concentration in the range of 5–200 μM and a detection limit down to 2.8 μM ($S/N=3$) were achieved. The specificity and application in real sample were validated. Furthermore, a verification test of thrombin detection in the same strategy illustrated its feasibility for not only small molecule but also biomacromolecule. With the advantage of high-throughput, easy operation, high specificity, the colorimetric assay based on ELAA requiring a single aptamer sequence opens up a new approach for detecting different kinds of targets via specific affinity recognition among target and suitably cleaved aptamer fragments.

1. Introduction

Aptamers are synthetic nucleic acid sequences selected by systematic evolution of ligands by exponential enrichment(SELEX) [1,2]. They can bind their respective targets ranging from small inorganic or organic substances to even proteins or cells. Aptamers have attracted increasing attention due to their advantages of high specificity and affinity, remarkable stability, easy modification and reproducibility. As biological recognition elements, aptamers have played an important role in establishing different kinds of biosensors [3]. A number of aptasensors based on various transducer technologies, such as electrochemistry [4–6] fluorescence [7–9], colorimetry [10,11], chemiluminescence [12], etc., have been explored in recent years.

Redox probes [5], nanoparticles such as Pt nanoparticles [13] and quantum dots [14,15], enzymes [16], peroxidase-like DNAzyme [17] were adopted as reporting groups to build different kinds of electrochemical aptasensors. Also, a few different fluorescence-based signal transduction mechanisms, such as direct fluorophore labeling [18], fluorescence resonance energy transfer (FRET) [19], fluorescence quenching [20], and fluorescence anisotropy [21,22] in combination with various labeling strategies of aptamers contribute to simple, rapid, sensitive and specific fluorescent aptasensors. Different amplification methods such as rolling circle amplification [23] and an autonomous aptamer-based machine based on polymerase/dNTPs [24] have been developed to obtain a higher sensitivity or a lower detection limit. Colorimetric aptasensors, probably not as sensitive as the two types described above, have a remarkable advantage such as convenience in that the signal readout requires only simple instruments or even no instrumentation when read with the naked eye. Many reporting groups such as dye molecules [10], gold nanoparticles and QDs [25,26], horseradish peroxidase (HRP) [27], an aptamer cross-linked hydrogel [28] have been used to give a colorimetric signal readout. Combined with the color reaction of peroxidase-like DNAzyme [17], logic gates [29] and DNAzyme cascades [30] were well designed based on encoding G-Quadruplex sequences and aptamer sequences or DNAzyme sequences for the detection of a variety of targets.

Enzyme linked aptamer assay (ELAA), is a variant of ELISA (enzyme linked immunosorbent assay) using aptamers instead of antibodies. Direct and indirect competitive ELAA have been developed via labeled targets [31,32]. Noncompetitive methods are also explored mainly based on the following strategies: aptamer/target/aptamer type [33,34]; antibody/target/aptamer

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sandwich type [35,36]; aptamer fragment/target/aptamer fragment type [16]. Competitive methods need to label the target, which might affect intrinsic affinity of aptamer and target. For noncompetitive methods, a target which can interact with two aptamers is needed in the aptamer/target/aptamer type, and antibody is used for the formation of sandwich structure in antibody/target/aptamer type. These demands limit the further development of analytical assays based on ELAA. Cai et. al. [12] reported a protein aptasensor based on ELAA via base-stacking enhanced by the stem loop configuration. When the approach was used to detect small molecules such as adenosine and Hg^{2+}, the stem–loop configuration and base-stacking were not enough to stabilize the capture via the tail. T4 DNA ligase was adopted to induce a ligation reaction, which undoubtedly led to cost increase and tedious operation. Based on self-assembly into supramolecular complexes via base-stacking and affinity interaction in the presence of target and two cleaved parts of an aptamer, we developed a novel type, which required only a single aptamer, for ELAA in previous work [16].

Herein, taking advantageous of ELAA based on the self-assembly of an aptamer fragment/target complex, we developed a high-throughput, simple, and practicable colorimetric aptasensor (Scheme 1). Cocaine, which is a strong central nervous system addictive stimulant drug, was chosen as a model target. It can increase the level of dopamine in the reward circuit of the brain. Abusing cocaine has a variety of psychological and physiological adverse effects. Therefore, developing a high-throughput, simple and practicable method for cocaine determination is needed in clinical diagnosis and law enforcement. In this work, the anti-cocaine aptamer was cleaved into two fragments, CoN and CoB. One fragment CoN was immobilized on a carboxyl N-oxysuccinnimide esters (NOS) modified DNA-BIND 96-well plate via labeled primary amines. When cocaine and two aptamer fragments (CoB and CoN) were present simultaneously, ternary complexes were able to form effectively. Then, streptavidin–HRP (SA–HRP) was conjugated to biotin. The catalytic oxidation of TMB–H_2O_2 (TMB: 4, 4′-diamino-3, 3′, 5, 5′-tetramethylbiphenyl) substrates by HRP led to the generation of color changes, which provided the readout responses for the assay. The sensor was applied to detect cocaine in human serum. Thrombin detection was also tested using a similar strategy to demonstrate the general applicability of the sensor for biomacromolecules. In the microplate colorimetric assay, a large numbers of samples can be detected at the same time. The small-volume well which served as both reaction and detection place can greatly reduce the quantities of samples or reagents required, and the stability and parallelism are satisfactory in this assay. As the assay process is similar to ELISA, it is simple to handle and washing steps provide the possibility of obtaining a low background. Although the analytical process is not quick, the relative handing time per sample can be decreased by several minutes due to the use of 96-well plate. On the other hand, the introduction of the novel ELAA type using only a single aptamer based on cleaving and self-assembling into microplate is expected to open up a new approach for detecting different kinds of targets. The assay shows aptamers can be widely used as chemical antibodies in their true sense.

2. Experimental

2.1. Reagents and apparatus

All oligonucleotides were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and have the following sequences:

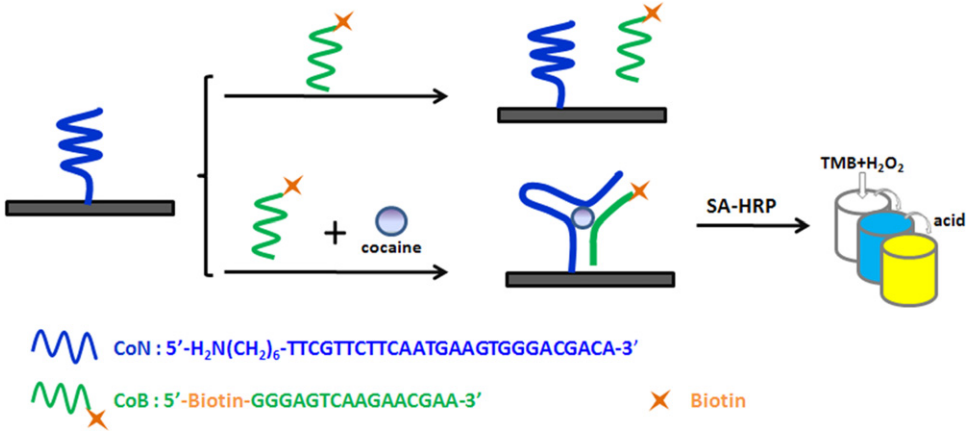
5′-NH_2–$(CH_2)_6$–TTCGTTCTTCAATGAAGTGGGACGACA-3′, CoN
5′-Biotin–GGGAGTCAAGAACGAA-3′, CoB
5′-Biotin–GGGAGTCAAGAACG-3′, CoB-2
5′-Biotin–GGGAGTCAAGAA-3′, CoB-4
5′-Biotin–GGGAGTCAAG-3′, CoB-6
5′-Biotin–GGGAGTCA-3′, CoB-8
5′- NH_2–$(CH_2)_6$–AAAAAAAAAAAGACGGTTGGTG -3′, TBA-N
5′-TGGTTGGGTCT–Biotin-3′, TBA-B

SA–HRP was purchased from Sigma-Aldrich. TMB · 2HCl was bought from Ameresco (USA). Bovine serum albumin (BSA) was purchased from Zhongshenghuamei Beijing Technology Co., Ltd (Beijing, China). Cocaine hydrochloride was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Morphine hydrochloride was obtained from Qinghai pharmaceutical factory (Qinghai, China). Human alpha thrombin was purchased from Enzyme Research Laboratories, Ltd (USA). All other reagents were of analytical reagent grade and the water used was purified by a Millipore Milli-XQ system.

DNA-BIND 96-well plates were purchased from Corning Incorporated (USA). Absorption signals were recorded on Thermo scientific Multiskan FC Microplate Photometer. Beckman GS-15R centrifuge was used for ultrafiltration.

2.2. Colorimetric detection of cocaine

Amino-modified CoN was dissolved in oligo binding buffer (0.05 M NaH_2PO_4–Na_2HPO_4, pH 8.5). 100 μL/well CoN solutions



Scheme 1. Schematic representation of the sensing procedure for colorimetric detection of cocaine based on ELAA on 96-well plate.

were added into a DNA-BIND 96-well plate at a concentration of 50 pmol/well. The wells were coated with reactive NOS, which could covalently couple with amino modified oligonucleotide. After 1 h incubation at 37 °C, the plate was washed three times by filling each well with 200 μL wash buffer (0.1 M NaH_2PO_4–Na_2HPO_4, pH 7.4, 5 mM KCl, 5 mM $MgCl_2$, 0.05% Tween 20) to remove unbound CoN. The wells were blocked by 1 h incubation with 200 μL oligo binding buffer containing 5% BSA at 37 °C and washed three times as described above. Different amounts of cocaine and CoB in 100 μL of phosphate buffer (0.1 M NaH_2PO_4–Na_2HPO_4, pH 7.4, 5 mM KCl, 5 mM $MgCl_2$) were then added into each well and incubated for another 1 h at 25 °C. After washing, 100 μL/well 4 μg/mL SA–HRP (in phosphate buffer) was added. Following 30 min reaction at 25 °C, 200 μL/well TMB–H_2O_2 working solutions (0.12 mg/mL TMB·2HCl, 18 mM H_2O_2, 0.1 M NaH_2PO_4–Na_2HPO_4, pH 6.0) were added after washing. The mixture was kept for 40 min at 25°C without direct light exposure, and terminated by addition of 50 μL 2 M H_2SO_4.

2.3. Serum samples treatments

Human serum was obtained from the local hospital and subjected to ultrafiltration by loading into a centrifugal filtration tube (MWCO = 3 kDa, Millipore) at 10,000 rpm for 90 min. Different amounts of cocaine stock solution, a certain amount of CoB solution and pretreated human serum were intensively mixed to obtain final serum samples: 0, 10, 50, 100 μM cocaine; 5 pmoL/well CoB; 10% human serum. Phosphate buffer (0.1 M NaH_2PO_4–Na_2HPO_4, pH 7.4, 5 mM KCl, 5 mM $MgCl_2$) was used in the whole treatment step.

2.4. Colorimetric detection of thrombin

Amino-modified TBA-N was dissolved in oligo binding buffer (50 mM NaH_2PO_4–Na_2HPO_4, pH 8.5). 100 μL/well TBA-N solutions were added into a DNA-BIND 96-well plate and incubated at 37 °C for 1 h. After the blocking process, different concentrations of thrombin, hemin and TBA-B in 100 μL of 20 mM Tris–HCl buffer (pH 7.3, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) were added and made to react at 4 °C for 6 h. Washing, blocking, streptavidin–biotin conjugation, TMB–H_2O_2 chromogenic reaction and the termination process were carried out the same way as described above.

3. Results and discussion

3.1. Sensing mechanisms of the aptasensor

Aptamers can specifically bind to target and fold into stable stem–loop structure. As shown in Scheme 1, an aptasensor for the colorimetric detection of cocaine on 96-well plate was established by ELAA. The anti-cocaine aptamer was cleaved into two parts: CoN, labeled with amino at the 5′- end; CoB, labeled with biotin at the 5′ end. The surface of the DNA-BIND 96-well plate is coated with reactive NOS, and can react with the primary amine of CoN. Washing steps and a BSA blocking process were carried out in order to obtain a low background signal by eliminating non-specific adsorption (Fig. 1a). In the absence of cocaine, the immobilized $CoNH_2$ olignucleotide could not form complexes effectively with CoB. The slight signal increase was due to the hybridization of the two strands by themselves to some extent (Fig. 1b). However, in the simultaneous presence of cocaine and CoB, the signal obtained increased apparently (Fig. 1c), proving that CoN/cocaine/CoB ternary complexes could self-assemble via the affinity interaction among them. The biotin labeled at the 5′-end of CoB adopted a suitable space orientation away from surface of plate, which was propitious to react with SA–HRP. A peroxidase-labeled aptasensor was formed via linking SA–HRP to the ternary complexes. Because of the high catalytic activity of HRP for the HRP–TMB–H_2O_2 colorimetric system, the signal which was related to the quantity of ternary complexes, further related to the concentration of target cocaine, could be obtained.

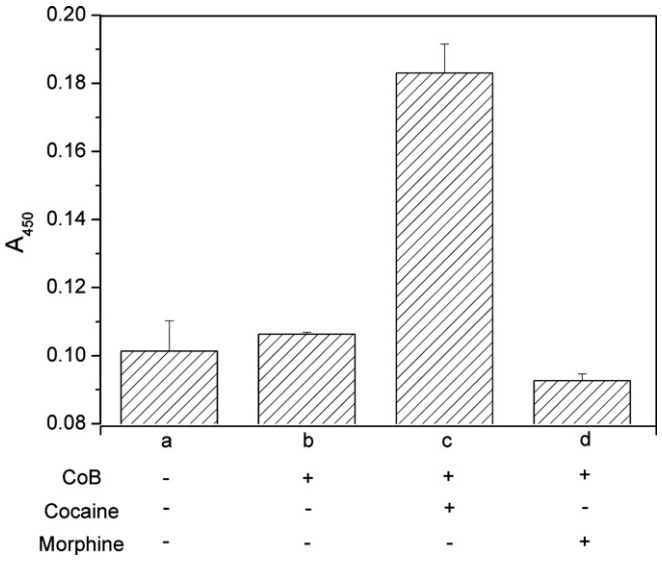


Fig. 1. Colorimetric signals of specificity investigation. Experimental conditions: 50 pmol/well CoN, 5 pmol/well CoB, 100 μM cocaine, 1 mM morphine. Error bars: SD, $n=3$.

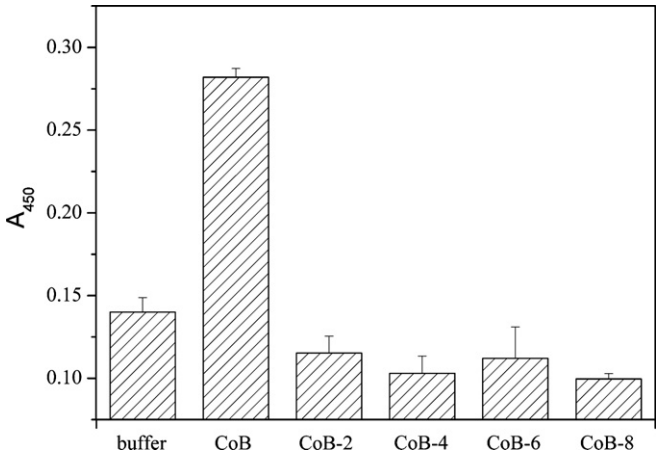


Fig. 2. Responses of colorimetric detection with different lengths of DNA oligonucleotides (CoB, CoB-2, CoB-4, CoB-6, CoB-8). Experimental conditions: 50 pmol/well CoN, 5 pmol/well each DNA oligonucleotide, 100 μM cocaine. Error bars: SD, $n=3$.

Control experiments were performed to ensure that the colorimetric signals obtained were due to the formation of aptamer fragments/cocaine complexes. A series of DNA oligonucleotides which had two bases subtracted at the 3′-end of CoB, in turn, were named CoB-2, CoB-4, CoB-6 and CoB-8. These DNA sequences were used to perform the control investigation. As shown in Fig. 2, comparing with the complete sequence CoB, the DNA oligonucleotide sequences which had bases lacking led to low colorimetric signals which were even lower than the blank (buffer instead of DNA sequences). The results showed that sequences lacking bases could not form ternary complexes with CoN and the target cocaine. The lack of bases reduced the base

pairs of the stem–loop structure and decreased the contribution of base pairing and coaxial stacking interaction to the stability of ternary complexes [37]. The experiment indicated that only suitable aptamer sequences could ensure the effective formation of ternary aptamer fragments/target complexes. Control investigations validated that the formation of ternary complexes was the key to qualitative and quantitative detection.

We further investigated the aptamer binding reaction with anesthetic antalgic morphine to evaluate the specificity of the aptasensor to cocaine. Although the concentration of morphine (1 mM) was 10 times to that of cocaine (0.1 mM), no signal increase was obtained (Fig. 1d). The result indicated that the aptasensor was specific to cocaine, which was the benefit of the specific stem–loop structure of the aptamer and the high affinity to form aptamer-target ternary complexes.

3.2. Optimization of experimental conditions

In order to obtain optimal conditions of the cocaine aptasensor, several related parameters were investigated, including the concentration of CoB, the incubation time for the formation of aptamer fragments/cocaine ternary complexes and the response time for colorimetric detection.

The quality of CoN immobilized on the surface of wells was primary to the formation of ternary complexes. Increasing the concentration of CoN in the binding process, namely raising the coverage degree of CoN, was propitious to form ternary complexes; however, taking steric hindrance and configuration into consideration, the excessively high surface density might affect the formation of complexes and lead to an unobvious response. According to the references [12,38], binding conditions were selected as 50 pmol/well CoN incubation for 1 h at 37 °C. BSA could eliminate the nonspecific adsorption and block remaining NOS groups on the solid surface which might react with the amino groups of streptavidin or HRP. The process of blocking could reach a plateau after relative rapid growth at beginning. Therefore, the blocking time of 1 h was sufficient.

There was a chemical equilibrium among the three components, thus, the concentration of CoB was another important factor in the formation of supramolecular complexes. A low concentration of CoB was not favorable for the complex formation in the well but a high concentration of CoB could lead to a high background due to hybridization of the two strands themselves when cocaine was absent. Different amounts of CoB solution (without cocaine) were added and incubated with CoN, which had been linked to the wells. When the concentrations were 1, 2, and 5 pmol/well, the absorbances at 450 nm (A_{450}) were low and there were no obvious variations. When the concentrations were greater than 5 pmol/well, a sharp increase of A_{450} was observed (Fig. 3(A)). As a complete anti-cocaine aptamer was cleaved into two parts: CoB and CoN, a duplex binding equilibrium involving complementary pairing of the bases might coexist with the chemical equilibrium of aptamer fragments/target ternary complexes. The duplex binding could allow the labeled biotin to capture SA–HRP and lead to the high background. Therefore, 5 pmol/well CoB was considered to be an ideal concentration in the assay.

Incubation time for the formation of ternary complexes was another important factor to affect the sensitivity of the assay. As shown in Fig. 3(B), the response increased with increasing incubation time within 60 min and then reached to equilibrium after 60 min. The results showed that 1 h incubation time was enough for the determination of cocaine in the assay.

A HRP–TMB–H_2O_2 colorimetric system was used as signal readout of the aptasensor designed. A kinetic experiment was performed to obtain the optimum response time for the chromogenic reaction. The signals of A_{450}, which were shown in Fig. 3(C), increased rapidly at the beginning and reached a plateau at 40 min. Hence, 40 min was selected for the colorimetric responding process.

3.3. The calibration curve of cocaine detection

The quantitative behavior of the cocaine colorimetric aptasensor was investigated. Under optimal conditions, different amounts of cocaine and a certain amount of CoB were added into wells to form ternary complexes with CoN followed by a HRP–TMB–H_2O_2 colorimetric detection. In Fig. 4(A), the color changes depend upon the concentrations of cocaine which can be discriminated by the naked eye. Fig. 4(B) shows the percentage changes of the absorption value of the aptasensor upon detecting different concentrations of cocaine. As the concentration of cocaine increased, A_{450} increased significantly. There was a good linear relationship between signal gain % $((A_{450}-A_{450-\mathrm{blank}})/A_{450-\mathrm{blank}})$ and cocaine concentrations ranging from 5 μM to 200 μM (signal gain %$=6.91+0.899\ C_{\mathrm{cocaine}}$, $r^2=0.985$). The detection limit was estimated to be 2.8 μM at a signal to noise ratio of 3. Complementary pairing of the bases to stabilize the stem–loop structures and sufficient bases length were two necessary factors to form the hydrophobic surfaces and negative charges accumulated three-way junction [8]. The anti-cocaine aptamer, which has the same sequence to the one adopted in our assay, has been reported to have dissociation constants ranging from 10^{-7} M to 10^{-4} M detected by various experimental methods [7,8,13,39]. K_d is generally identified as 5 μM [7]. Because of the low affinity capacity between the target and the aptamer, the detection limit was relatively high for these kinds of aptasensor without feasible signal amplification. The sensitivity of the assay we designed was similar to or better than many previous cocaine aptasensors using fluorescent [7,8,24], colorimetric [10,26], electrochemical [4,5,14] and other assays [13,40]with or without signal amplification process. Given an overall consideration of many obvious advantages, such as low background, simple operation, convenience, high-throughput and relative short handling time of per sample, the sensitivity and detection range we obtained for quantitative analysis were satisfied.

3.4. Real sample analysis

In order to verify the feasibility of the cocaine colorimetric assay for practical application, we analyzed the performance of the aptasensor in diluted serum samples. Different concentrations of cocaine were added into 10-fold diluted serum samples, and the colorimetric responses were investigated in comparison with the responses of cocaine detected in buffer solution. As the results presented in Table 1 indicate, the responses in diluted serum samples showed a good coherence to those obtained in buffer solution, which revealed that the cocaine aptasensor can be used reliably in the complicated serum matrix.

3.5. Generalization of the aptasensor based on ELAA

In order to test the general applicability of the sensor we designed, thrombin was chosen as the model to validate whether the scheme could be extended to detect biomacromolecules. Thrombin is an essential serine protease in the blood coagulation cascade, and the detection of thrombin is needed in diagnosis and therapeutics. The 15-base anti-thrombin aptamer was split into two parts, TBA-N and TBA-B [41,42]. To avoid the influence of the substrate on the formation of aptamer fragments/biomacromolecule, a linker sequence consisting of 10 adenosines was added to the end of the anti-thrombin aptamer fragment (TBA-N). At first,

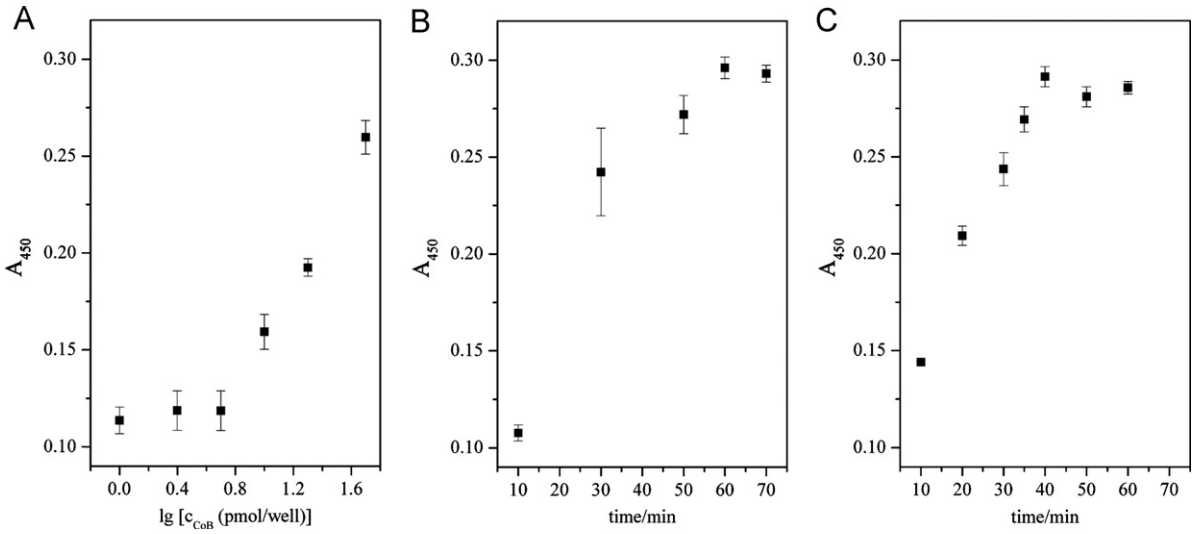


Fig. 3. (A) Responses of cocaine aptasensor with different concentrations of CoB: 1, 2, 5, 10, 20, 50 pmol/well without cocaine. Experimental conditions: 50 pmol/well CoN, 200 μL/well TMB–H_2O_2 working solution. (B) Time curve for the formation of aptamer fragments/cocaine ternary complexes. Experimental conditions: 50 pmol/well CoN, 5 pmol/well CoB, 100 μM cocaine, 200 μL/well TMB–H_2O_2 working solution. (C) The time-dependent signal responses for HRP–H_2O_2–TMB colorimetric reaction. Experimental conditions: 50 pmol/well CoN, 5 pmol/well CoB, 100 μM cocaine, 200 μL/well TMB–H_2O_2 working solution. Error bars: SD, $n=3$.

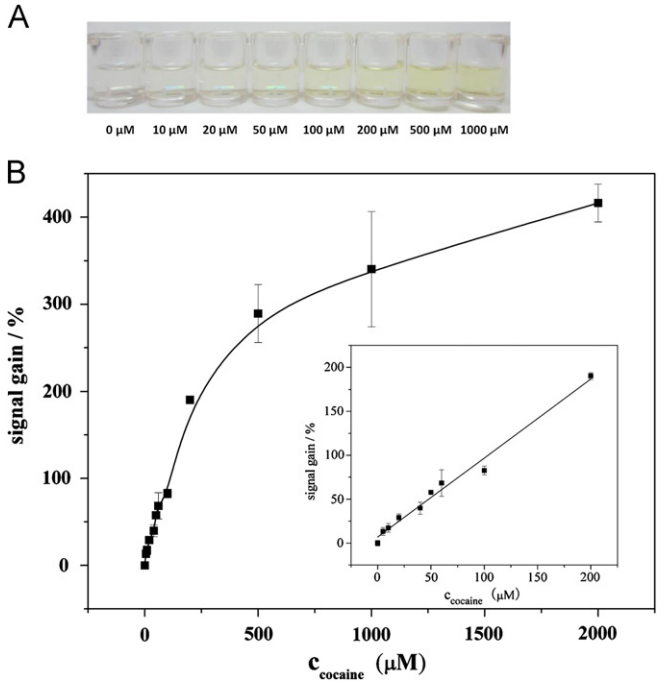


Fig. 4. (A) Photograph of cocaine concentration detection in phosphate buffer. (B) The corresponding calibration plot of the signal gain vs. $C_{cocaine}$. Inset in B: linear part of the plot in B ranging from 5 μM to 200 μM. The experiment was performed under optimal conditions. Error bars: SD, $n=3$.

the amino modified TBA-N sequence was linked to a 96-well plate through an amide bond. Then, thrombin and the other part of the biotin labeled TBA-B sequence were added (Scheme s1, see the supplementary material). In the presence of thrombin and K^+, the two parts of the sequences can fold into a G-quadruplex structure which in turn allows the conjugation of TBA-N/TBA-B/thrombin complexes and SA–HRP. Hemin can bind G-quadruplex structures to form peroxidase mimic DNAzyme. Therefore, the thrombin/TBA/hemin supramolecular complex can represent an enhancement in catalytic ability [43]. We investigated the sensors in two modes based on a cleaved aptamer type of ELAA: TBA-N/TBA-B/thrombin and TBA-N/TBA-B/thrombin/hemin. No significant signal increase

Table 1
Cocaine concentration detection in phosphate buffer and 10% diluted human serum.

	Phosphate buffer A_{450}	**10% Human serum A'_{450}**	**Recovery** $[A'_{450}]/[A_{450}]$ (%)
$C_{cocaine}=0$ μM	0.099(±0.004)	0.099(±0.006)	/
$C_{cocaine}=10$ μM	0.114(±0.001)	0.107(±0.008)	93.9
$C_{cocaine}=50$ μM	0.156(±0.013)	0.143(±0.014)	91.7
$C_{cocaine}=100$ μM	0.200(±0.005)	0.192(±0.019)	96

A_{450} represents the signal of the sensor after detection cocaine in phosphate buffer and A_{450} represents the signal detected in human serum. Recovery means $[A'_{450}]/[A_{450}]$.

was obtained in the TBA-N/TBA-B/thrombin mode. However, the absorbance of the latter mode, with the assistance of hemin, was enhanced upon the addition of different concentrations of thrombin. The colorimetric intensity gradient correlated positively with the concentration gradient of thrombin. As shown in Fig. S1, 10 μM bovine serum albumin (BSA) showed no interference, indicating that the anti-thrombin aptamer showed good specificity for thrombin only. The low association constant of the slow reaction equilibrium process and small quantity of TBA-N/TBA-B/thrombin ternary complexes formed in the 96-well plate might lead to the results of the previous mode. On the other hand, the addition of hemin might shift the equilibrium towards the formation of TBA-N/TBA-B/thrombin complexes. We also tested the strategy in the absence of SA–HRP (The biotin/avidin conjugation process was left out.) As the peroxidase-like activity of hemin/G-quadruplex structure, color changes were observed when thrombin was added in a homogenous solution system, whereas no significant signal difference was obtained in a heterogenous system. The reasons might be the limited quantity of immobilized aptamer fragments, the discrepancy in catalytic ability of HRP and G-quadruplex mimic enzyme; the different interactive efficiency of homogeneous and heterogenous reactions. The success of thrombin detection indicated the feasibility of this sensor to be used for biomacromolecule detection. As illustrated in Fig. S1, 10 nM thrombin can trigger a marked signal readout. The sensor shows the dynamic range from 0 to 200 nM for thrombin with the lowest detectable concentration of 10 nM at present. We expect that a satisfactory detection limit might be achieved via a systematic optimization process.

4. Conclusion

With the advantage of a self-assemble aptamer fragment/target complex and HRP colorimetric system, we have demonstrated a high-throughput, simple, and practicable aptasensor based on ELAA only requiring a single aptamer sequence. In order to test the feasibility of the assay, cocaine was selected as a model molecule. A linear response ranging from 5 μM to 200 μM and a detection limit of 2.8 μM were obtained. The specificity and application in biologic fluid were evaluated to be satisfactory. The successful detection of thrombin in a preliminary verification test illustrated the feasibility of the ELAA sensor for both small molecule and biomacromolecules. Further improving the sensitivity to meet higher demand, the amplification process in aqueous phase, which might gain more obvious response, can be designed into the platform like gold nanoparticles, DNAzyme cascades, smart nanomaterials or mimicking enzyme. As the novel ELAA type using only a single aptamer was introduced into microplate, we anticipate it to be a generalized method for detecting a large number of targets in bioanalysis and drug control.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/http://dx.doi.org/10.1016/j.talanta.2012.11.018.

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