Analyst

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d0an00675k

A cost-effective fluorescence biosensor for cocaine based on a "mix-and-detect" strategy†

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Received 6th April 2020,
Accepted 13th May 2020

DOI: 10.1039/d0an00675k

rsc.li/analyst

The efficient detection of illicit drugs such as cocaine continues to be important for the fight against drug trafficking. Herein, we report a one-step method for rapid and specific cocaine detection. The method is based on our finding that small-molecule Thioflavin T (ThT) can act as a fluorescence indicator, which can be bonded with the anti-cocaine aptamer (MNS-4.1) to generate an enhanced fluorescence signal. More interestingly, upon cocaine binding, the intercalated ThT can be replaced, causing a drastic fluorescence reduction. We further optimized the sequence of MNS-4.1 and a new anti-cocaine aptamer (coc.ap2-GC) was obtained. This aptamer showed a higher affinity to both ligands, which increased the ThT binding fluorescence intensity and showed the highest quenching efficiency. Based on the fluorescence change induced by competitive binding, cocaine detection could be accomplished by a "mix-and-detect" strategy within seconds. Such a label-free method exhibits high sensitivity to cocaine with a low detection limit of 250 nM. Moreover, the practical sample analysis (2.5% human urine and saliva) also exhibits good precision and high sensitivity.

1. Introduction

Cocaine is a central nervous system addictive drug. Many dangers such as anxiety, cardiovascular problems, mood disturbances, insomnia, loss of appetite, and violent behavior can be caused by abusing cocaine.[1–4] With the drug market expanding, global cocaine production (estimated at 1410 tons) reached its highest level in history in 2016, posing multiple challenges in many aspects.[5] Therefore, developing a reliable screening method for the rapid and specific detection of cocaine is extremely important for illicit drug control around the world. Until now, various methods have been proposed, including the most popular surface-enhanced Raman spectroscopy (SERS),[6,7] and colloidal gold immunochromatographic assay (GICA).[8] Some of them are suitable for in-field sample evaluation, but they are still limited due to obstacles such as substantial background interference induced by biological samples and poor specificity of antibodies for small molecular targets. They are prone to cross-reactivity leading to false-positive results.[9,10]

In the past decades, aptamer-based sensors for cocaine detection have attracted considerable attention because of the inherent properties of aptamers. Aptamers are single-stranded nucleic acids that are selected by an *in vitro* evolution process with high affinity and selectivity for target molecules.[11,12] They show various advantages such as flexible design, low cost to produce, high chemical stability, and easy chemical modification.[13–15] Up to now, many aptamer-based sensors for the rapid detection of cocaine have been reported.[16–18] Most of them are based on the mechanism of target-induced conformational change.[19,20] For example, the MNS-4.1 cocaine binding aptamer[21,22] could remain in an unfolded structure in the absence of cocaine, while, in the presence of cocaine, it folds into a typical specific three-way junction structure that binds the target. However, methods based on target-induced conformational changes are often difficult to control. Sometimes, they need to label or modify chemical probes with a reporter for the signal readout.[23,24] Sometimes, they need to truncate or intervene in the sequence to destabilize the aptamer so that it consists of an equilibrium state containing both unfolded and folded structures.[25] Those processes will result in high background and notably reduce target binding affinity, which is not conducive to the improvement of detection performance.

As a solution to this problem, researchers considered that if an aptamer would bind both a dye and the target analyte, then

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†Electronic supplementary information (ESI) available: Fig. S1–S6 and Tables S1–S3. See DOI: 10.1039/d0an00675k

the binding of the target analyte might alter the microenvironment of the dye and produce a visible signal of that event. Xiao's group[26] found a special fluorogenic dye, 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND). This dye can compete with cocaine to bind with cocaine aptamer 38-GC. In the absence of cocaine, ATMND quickly binds with 38-GC and thereby quenches its fluorescence. In the presence of cocaine, cocaine displaces ATMND from the binding domain and then the fluorescence recovers.[26] Based on this finding, a typical "mix and detect" biosensor was constructed, wherein, 38-GC is a fully-folded aptamer for cocaine, which keeps the binding affinity at maximum. Therefore, it can detect as low as 200 nM cocaine in seconds. Comparing with other cocaine detection methods, the "mix and detect" biosensor is really simple, rapid, and effective. This strategy was of particular interest to us, but only few dyes have been reported.[26–28]

In this work, we found another fluorescent dye Thioflavin T (ThT), which can bind with cocaine aptamer MNS-4.1 and generate an enhanced fluorescence signal. ThT is a commercial dye with a low molecular weight. It is very cheap, stable, and water-soluble.[29–31] We also found that cocaine can displace the bound ThT efficiently, resulting in rapid fluorescence quenching. In addition, this binding and displacement can be achieved in seconds. Based on these findings, we proposed a competitive mechanism and verified it. Then, a new mutant (cocap2-GC) of MNS-4.1 was derived to further improve the binding affinity with cocaine and can achieve the best quenching efficiency. We constructed a "mix and detect" assay for the rapid detection of cocaine at a concentration of 250 nM within only seconds. It was about 40-fold lower than that of the target-induced conformational change method,[20] but is cost-effective and straight forward.[32,33] Most importantly, the entire reaction process does not require incubation or stabilization time. In addition, our sensor successfully achieves the immediate detection of cocaine in body fluids.

2. Experimental section

2.1 Materials

All aptamer DNAs used in this work were synthesized and purified without any modifications by Sangon Biotech Inc. (Shanghai, China). Thioflavin T (ThT) and SYBR Green I (10 000×) were ordered from Sigma-Aldrich (St Louis, MO, USA) and freshly prepared before use. Tris, sodium chloride, ATP and other reagents were purchased from Aladdin (Shanghai, China). Cocaine, theophylline, and caffeine were obtained from Beijing Institute for Drug Control (Beijing, China). The healthy human urine and serum samples were provided by Dongguan Tungwah Hospital. The saliva sample was obtained from our laboratory staff, and for these samples, informed consent was obtained from all human subjects. Other reagents were used as received without further purification. All aqueous solutions were prepared with a Milli-Q water system (resistance >18 MΩ cm^{-1}). All buffer solutions and ultrapure water were sterilized and used throughout experiments.

2.2. Fluorescence measurements

The reaction sample (150 μL) was mixed with 10.0 μL of 15 μM cocaine aptamer oligomer, 10.0 μL of 15 μM ThT, and 7.5 μL of various concentrations of cocaine in 25 mM Tris buffer (pH = 7.40, 50 mM KCl), and then followed by fluorescence measurement. Fluorescence experiments were performed on an Edinburgh FS5 fluorescence spectrometer in the emission wavelength from 450 to 600 nm with 435 nm excitation. The slits of excitation and emission were set at 5.0 nm.

2.3. Binding assays

The stoichiometries of ThT/coc.ap2-GC bindings were determined *via* the continuous variation analysis (Job plots) method.[34] Briefly, different concentrations (from 0 to 1.0 μM) of coc.ap2-GC were dissolved in the Tris-HCl buffer, and then different concentrations (from 1.0 to 0 μM) were mixed to read the fluorescence intensity at 484 nm with an excitation wavelength of 435 nm. It is worth noting that we must control the sum of ThT and cocaine concentrations to 1 μM in each sample.

Isothermal titration calorimetry (ITC) experiments were performed with a Nano ITC instrument (TA, USA). Aptamer and ThT (or cocaine) samples were prepared with Tris buffer (25 mM Tris, pH = 7.40, 50 mM KCl). The concentration of the aptamer in the cell was kept at 40 μM, while the titrant (cocaine or ThT) in the syringe was 300 or 500 μM. We introduced 50 μL total injections 25 times, each time for 2 μL after a 0.4 μL purge injection. ITC experiments were performed at 25 °C. The raw data were analyzed by NanoAanlyze software.

2.4 UV-visible absorption spectroscopy

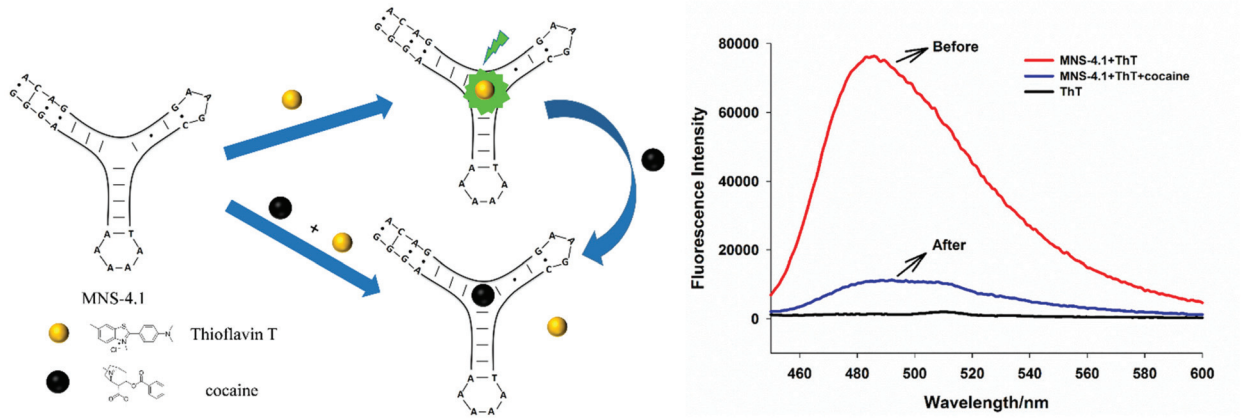
UV-vis absorption spectra were all recorded from 300 to 500 nm using a UV2700-vis spectrometer (SHIMADZU, Japan). The mixture solution (150 μL) contains 4 μM ThT, 2 μM coc. ap2-GC and different concentrations of cocaine in the Tris-HCl buffer. Each sample was scanned three times and averaged. Final data were subtracted from the blank absorption.

3. Results and discussion

3.1. The principle of cocaine detection

Recently, we discovered that ThT could act as a new fluorescence indicator for anti-cocaine aptamers. When we mixed 1 μM ThT with 200 nM aptamer MNS-4.1 (Scheme 1), a remarkable fluorescence enhancement can be obtained. Attractively, when cocaine was incubated with them, roughly 85% of the fluorophore was quenched. Both the enhancement and quenching can be achieved by mixing within seconds. Based on those findings, we proposed a "mix and detect" type fluorescence biosensor for cocaine detection.

We speculate that this biosensor may be based on a competition mechanism. As we know, ThT is a kind of benzothiazole dye with low fluorescence emission in its unbound state.[35] Any interaction that limits the rotation of a single C–C bond and planarizes ThT leads to a tremendous increase in fluorescence emission.[35–38] Recent research studies demonstrated that



Scheme 1 The principle of the "mix and detect" type fluorescence aptasensor for cocaine detection.

some specific DNA structures such as G-quadruplexes,[39] G-triplexes,[40] and DNA cavity sites,[41] provided an adequately sized cavity to limit ThT. Their bindings give rise to remarkable signal-on in fluorescence. In this paper, the cocaine binding aptamer can be folded into three helical stems constructed around a three-way junction pocket. The special three-way junction pocket may be the premier site for both ThT and target cocaine binding. Nevertheless, the binding affinity of target cocaine may be more robust than that of ThT. In the absence of cocaine, ThT may be rapidly bound to the cocaine aptamer, and the complexing with the aptamer dramatically enhances the fluorescence, but in the presence of cocaine, the target cocaine may cause ThT to rapidly compete or be replaced from the MNS-4.1 (Scheme 1). In order to obtain a biosensor with superior performance, we should further optimize the experimental conditions and verify this competitive mechanism.

3.2. Optimization

Firstly, we optimized the sequences of the anti-cocaine aptamer. Previous studies have suggested that MNS-4.1 has three noncanonical base pairs in stem 1, resulting in reduced efficiency of ThT and cocaine binding, and thereby resulting in a lower original fluorescence and the bad quenching efficiency.[22] The Neves group[42] has demonstrated that the cocaine binding aptamer consisting of three helical stems folded into a three-way junction. There was only one Watson–Crick base pair in stem 2, while stem 1 and stem 3 contain two putative noncanonical base pairs, respectively. Moreover, a properly sized cavity can result in an enormous increase in the fluorescence emission of ThT once it limits the rotation of a single C–C bond and planarizes ThT.[35–37,43]

We designed a series of aptamer variants by changing the putative noncanonical base pairs in three helical stems to Watson–Crick base pairs (as shown in Table S1†). Fig. 1 depicts the fluorescence intensity of ThT in the absence and presence of cocaine with various variants, and the percentages in the graph represent the fluorescence quenching efficiency of each variant. We observed that the coc.ap3 aptamer results in approximately 96% quenching while a few vary when only changing stem 2 (coc.ap4) or stem 3 (38-GC-2). The coc.ap3 aptamer was engineered by converting all non-classical base

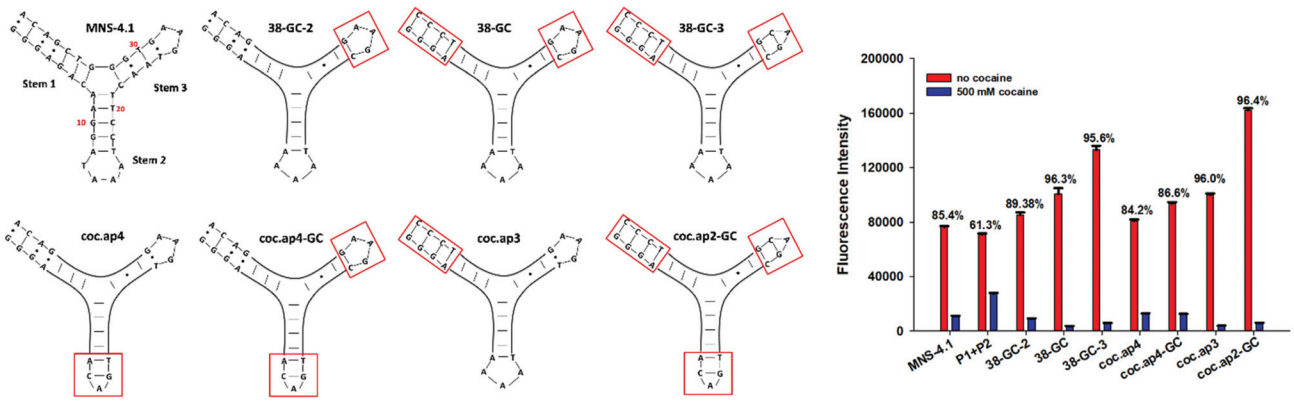


Fig. 1 Fluorescence intensity of ThT with different aptamer variants in the presence or absence of cocaine. The concentration of the variants, ThT, and cocaine, was 200 nM, 1 μM, and 500 μM, respectively. The sequences of P1 and P2 were 5′-AGC GGGAGACAAGAACGAA-3′ and 5′-TTCGTTCTTCAATGAAGTGGGTCGACA GCT-3′, respectively. Error bars represent the SDs of three measurements.

pairs in stem 1 into complementary pairs, because the cocaine-binding aptamer was wholly folded in the free form when in stem 1 with six complementary pairs.[42] Furthermore, we can find that the subsequent transformation of the G–T wobble pair in stem 3 of 38-GC to a matched G–C base pair further improves the quenching efficiency to 96.3%. The previous study suggested that having a matched G–C base pair in stem 3 can achieve maximal affinity.[26,42] Surprisingly, when all of the noncanonical base pairs in the three helical stems (coc.ap2-GC) were converted into complementary pairs, the fluorescence of ThT further enhanced dramatically. As demonstrated above, the mutant aptamer of cocap2-GC led to a further increase of ThT binding fluorescence intensity and the highest quenching efficiency. On the other hand, making changes to the three stems indicated that all of the three stems are not critical for ligand binding, corresponding to previous reports.[42] Since coc.ap2-GC was found to exhibit preferable fluorescence enhancement and quenching efficiency than other mutants, it was chosen for further sensor study.

Secondly, the concentration of ThT has to also be optimized. The concentration of ThT affects the initial fluorescence signal and the efficiency of quenching. With this in mind, we employed 200 nM coc.ap2-GC to optimize the concentration of ThT. As seen from Fig. S1† the fluorescence of ThT increases with increasing ThT concentration, up to 1.0 μM. Importantly, this concentration also can reach the highest quenching value.

Finally, we optimized the reaction time. Fig. 2 is a time-scan diagram of the changes in fluorescence intensity during aptamer-bound ThT and ThT replacement by cocaine. Free ThT has low fluorescence emission, but when ThT is rapidly bound by coc.ap2-GC, the fluorescence dramatically enhances and we did not observe any fluorescence change in one hour. Upon the addition of cocaine, we can observe that fluorescence was rapidly quenched and stabilized. It was clearly shown that coc.ap2-GC adopts a conformation favoring the interaction with cocaine rather than ThT. That is, ThT was replaced by cocaine. More interestingly, we found that the entire combination and replacement process was very rapid. In order to more clearly capture reaction as the whole competition process, we further monitored fluorescence changes during the binding and replacement process within one minute (Fig. S2†). We can observe that ThT binding to the aptamer only needs about 20 s and ThT replacement by cocaine use less time about a few seconds. From the above observations, since the displacement between ThT and cocaine was very stable, we can achieve the detection of cocaine within a few seconds.

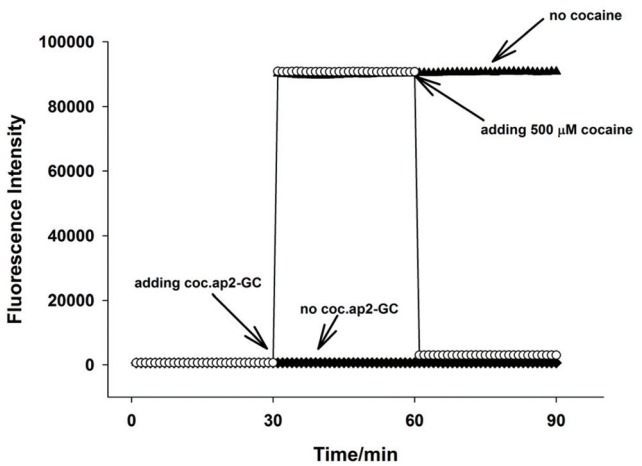


Fig. 2 Time course of ThT binding and release progress in the presence of coc.ap2-GC and cocaine. ThT has been bound to the coc.ap2-GC and result in vigorous fluorescence intensity. Subsequently, the ThT was released and rapidly quenched upon the addition of the cocaine. The concentration of the coc.ap2-GC, ThT and the cocaine was 200 nM, 1 μM, and 500 μM, respectively.

3.3. Competition mechanism between ThT and cocaine

The mechanism of this 'mix and detect' biosensor is more likely based on the competitive binding of the aptamer between ThT and cocaine. We assume that the hydrophobic three-way junction pocket of the aptamer is the binding site of both ligands. To verify this conjecture, we firstly examined the stoichiometries of ThT/coc.ap2-GC associations by using the continuous variation analysis (Job plots) method[34] (Fig. 3C). The result suggests a 1 : 1 interaction between ThT and the coc.ap2-GC. In other words, each coc.ap2-GC aptamer binds to one ThT molecule. Then we introduced a nucleotide mutation or switch (coc.ap2-GC-M1; M2, Fig. S3†) at the hydrophobic three-way junction pocket of coc.ap2-GC. It has been reported that the hydrophobic pocket is the crucial site for cocaine binding.[26] Any mutations to nucleotides can greatly change the binding affinity of the aptamer for cocaine.[26,44,45] Interestingly, we observed both low fluorescence enhancement and suboptimal cocaine-displacement quenching efficiency, indicating that both ligands shared the same binding site. Furthermore, we replaced ThT with a double-strand DNA indicator SYBR Green I and the test is shown in Fig. S4.† There is no noticeable signal change after the fluorescence of the SYBR Green I/coc.ap2-GC system. The addition of cocaine suggests that the ThT binding site is not the duplex stem of the aptamer. Finally, the binding affinity of ThT/coc.ap2-GC and cocaine/coc.ap2-GC was further investigated by using isothermal titration calorimetry (ITC). The results showed (Fig. 3A and Fig. S5†) that the binding in both cases was enthalpically driven and one-to-one. After correction for the heat of dilution, we obtained equilibrium dissociation constants (K_d) by using a single-site binding model.[26] The K_d for coc.ap2-GC binding to cocaine and ThT was 2.0 ± 0.6 μM, 3.7 ± 1.1 μM, respectively. The result indicated a tighter binding to cocaine, supporting our competitive mechanism.

To further verify this competitive mechanism, we utilized UV-vis to investigate the peak shift of the ThT/coc.ap2-GC complex with the different concentrations of cocaine. In Fig. 3B, we discovered the process of ThT replacement by cocaine *via* UV-vis spectroscopy measurement. Absorption of

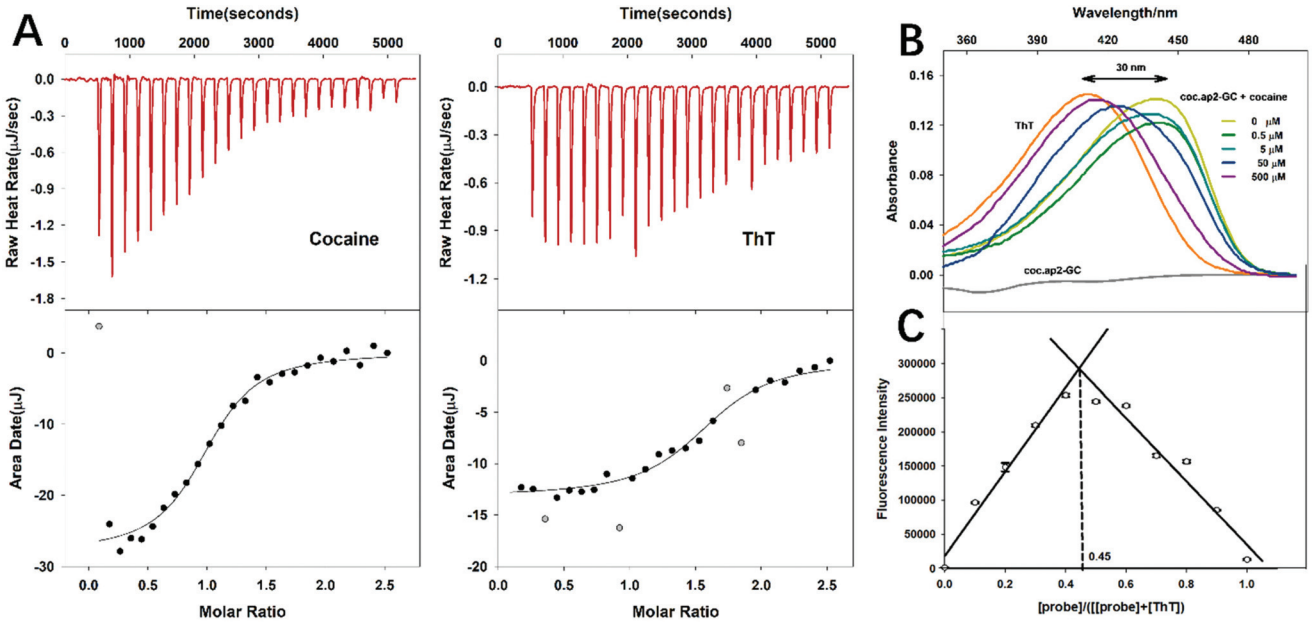


Fig. 3 (A) ITC data demonstrated that cocap2-GC binds both cocaine and ThT. The top is the heat generated from each injection of 300 μM cocaine or 300 μM ThT into 20 μM cocap2-GC aptamer solution. The bottom is the integrated heat plot after correcting for the heat of dilution. (B) UV-vis absorption spectra of free ThT and after increasing the concentrations of the cocaine in the ThT/coc.ap2-GC complex. (C) Job plots obtained from the fluorimetric analysis of mixtures of ThT with coc.ap2-GC ([ThT] + [coc.ap2-GC] = 1 μM) in Tris-HCl buffer. Error bars represent the SDs of three measurements.

the free ThT ligand was observed near 414 nm, which was consistent with the previous study.[29] Upon addition of coc.ap2-GC, a redshift to around 443 nm is observed. The formation of the ThT/coc.ap2-GC complex can result in preventing the rotation of the benzothiazole ring relative to the aminobenzene ring in the excited state.[46] As the concentration of cocaine increases, the absorption peak of ThT gradually restores to 414 nm, precisely indicating the replacement of ThT by cocaine. Therefore, the competitive mechanism (comparatively low affinity) enables the replacement of ThT, which will be useful in cocaine detection *via* employing ThT as a signal reporter.

3.4. Analytical performance for the detection of cocaine

Under the optimal conditions, the sensitivity of our aptasensor was investigated using the coc.ap2-GC/ThT complex with different concentrations of cocaine. As shown in Fig. 4A, with increasing concentration of cocaine, the fluorescence intensity decreases gradually. Therefore, it is a "signal off" aptasensor. Fig. 4B shows that there is a good linear relationship between the fluorescence signal change (F_0–F) and the logarithm of cocaine concentration, with a linear range of 1 μM to 500 μM for sensitive cocaine quantitation. The regression equation was $\Delta F = 50364 \log[\text{Ccocaine}] + 308\,315$ ($R = 0.9939$). The limit of detection was calculated to be 250 nM estimated at a signal-to-ratio of S/N > 3. To our knowledge, this detection limit is comparable to those of previously reported cocaine detection protocols (Table S2†).

3.5. The specificity and practical application of the cocaine aptasensor

To validate the selectivity of the ThT-based aptasensor, we challenged the assay against other small molecules, such as theophylline, adenosine monophosphate, adenosine triphosphate, caffeine, dopamine, glucose, and sucrose were used to replace cocaine to perform the experiments under the same conditions. As shown in Fig. 5, the fluorescence intensity of the ThT/coc.ap2-GC showed a dramatic decrease when introducing 500 μM cocaine, while the other small molecules even at 10-fold higher concentration than that used for cocaine induced very few changes in fluorescence intensity. These results indicate that our designed sensor exhibits good selectivity for cocaine detection, which is consistent with the previous reports of aptamer-based sensors (Table S2†). Such good selectivity may be relative to the native specificity of the aptamer toward its target.[18]

In order to evaluate the practicality and operability of our proposed method, we collected human saliva, urine and serum samples from healthy donors and diluted them with Tris-HCl buffer. We spiked different concentrations of cocaine into the diluted biological samples (2.5% v/v human urine and 2.5% v/v human saliva, 2.5% v/v human serum). As Fig. S6† shows, the linear range of the aptasensor measured in 2.5% urine was from 5 μM to 500 μM with a detection limit of 1.41 μM. Furthermore, we also achieved cocaine detection and exhibited excellent precision from 2.5 μM to 500 μM with a LOD of 1.15 μM in 2.5% saliva. The recovery experimental data

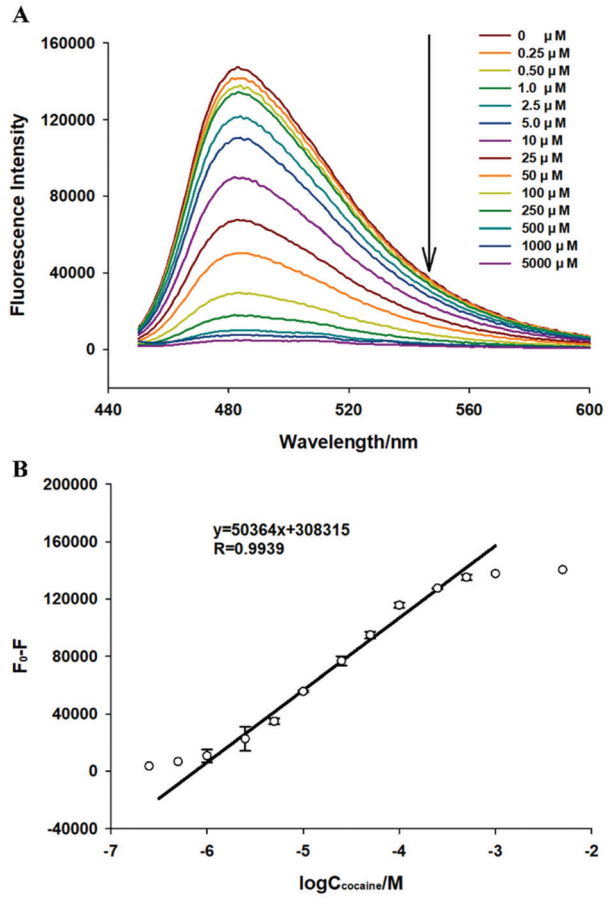


Fig. 4 (A) Fluorescence spectra of the ThT-based aptasensor toward different concentrations of cocaine. (B) A linear relationship between the fluorescence response change and the logarithm of cocaine concentrations. Fluorescence response change (F_0–F), where F_0 and F represent fluorescence intensity in the absence and presence of cocaine. Error bars represent the SDs of three measurements.

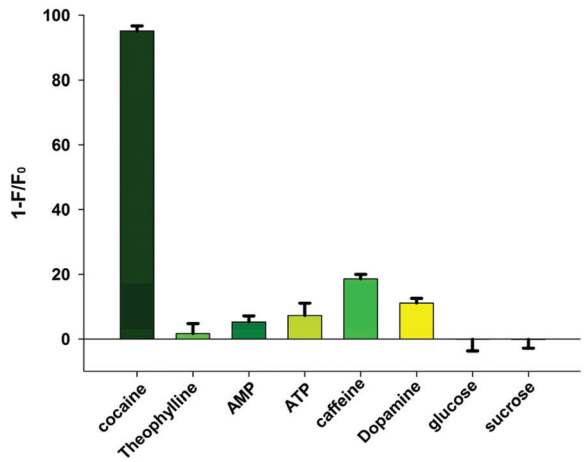


Fig. 5 The selectivity of the ThT-based sensor against target cocaine (500 μM), theophylline (1.0 mM), AMP (1.0 mM), ATP (500 μM), caffeine (500 μM), dopamine (500 μM), glucose (5.0 mM), and sucrose (5.0 mM). Error bars represent the SDs of three measurements.

are listed in Table S3.† Recoveries were found to range from 92.4% to 106.0%. In short, these experiments demonstrate that our ThT-based aptasensor could potentially be used to detect cocaine in complex systems.

4. Conclusions

In summary, we have constructed a one-step method for rapid and specific cocaine detection, which is based on a competition mechanism between the fluorogenic dye ThT and the cocaine target binding the cocaine aptamer. This is the first work using ThT as an efficient fluorescent ligand for the cocaine-binding aptamer. It is worth noting that a "mix-and-detect" step could accomplish cocaine detection, that is, the entire reaction progress can be achieved by mixing the ThT/coc.ap2-GC complex in a single tube without any stabilization or incubation time before the test. Furthermore, under room temperature, detection only requires seconds to achieve a linear range of 1–500 μM with a LOD of 250 nM. Compared with other cocaine sensors, it is low-cost and less time-consuming. More importantly, our sensor successfully achieves the immediate detection of cocaine in body fluids, which may have great potential for specific high-throughput on-site drug testing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We are grateful to the National Natural Science Foundation of China (Grant No. 21864004, 21964003 and 21405023) for their financial support of this work.

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