Direct fluorescence anisotropy assay for cocaine using tetramethylrhodamine-labeled aptamer

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Abstract Development of simple, sensitive, and rapid method for cocaine detection is important in medicine and drug abuse monitoring. Taking advantage of fluorescence anisotropy and aptamer, this study reports a direct fluorescence anisotropy (FA) assay for cocaine by employing an aptamer probe with tetramethylrhodamine (TMR) labeled on a specific position. The binding of cocaine and the aptamer causes a structure change of the TMR-labeled aptamer, leading to changes of the interaction between labeled TMR and adjacent G bases in aptamer sequence, so FA of TMR varies with increasing of cocaine. After screening different labeling positions of the aptamer, including thymine (T) bases and terminals of the aptamer, we obtained a favorable aptamer probe with TMR labeled on the 25th base T in the sequence, which exhibited sensitive and significant FA-decreasing responses upon cocaine. Under optimized assay conditions, this TMR-labeled aptamer allowed for direct FA detection of cocaine as low as 5 μ M. The maximum FA change reached about 0.086. This FA method also enabled the detection of cocaine spiked in diluted serum and urine samples, showing potential for applications.

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Introduction

Cocaine, known as benzyl ecgonine, is an alkaloid extracted from coca leaves, and it is used for asthma treatment and as local anesthetic for clinical surgery [1]. Cocaine is considered to be one of the most powerful stimulant drugs today because of its addictive properties and instantaneous, overwhelming effects on the central nervous system [2–4]. Illegal abuse of cocaine can trigger a series of psychosocial and social problems and damages for cardiovascular and neurological systems, or even death [4]. Hence, it is of great importance to develop simple, rapid, and highly sensitive cocaine detection methods for law enforcement, border protection, forensic science authorities, and clinical diagnostics.

Stojanovic et al. reported the DNA aptamer against cocaine [5, 6]. The discovery of aptamer against cocaine allows to develop aptamer-based sensors for cocaine [5, 6]. Aptamers are single-stranded oligonucleotides selected from the nucleic acid library, specifically binding targets with good binding affinity [7]. Aptamers have attractive features, such as ease of synthesis, facile labeling, binding-induced structure change, capability of sequence amplification by enzyme reaction, and so on [7–10]. Aptamer-based assays have been applied to a variety of targets in versatile detection formats [7–13]. Stojanovic et al. developed a fluorescence switch sensor for cocaine based on binding-induced conformation change of aptamer and fluorescence quenching [5, 6]. Hereafter, there have been an increasing number of aptamer-based methods for detecting cocaine [3, 5, 6, 8, 11, 14–16].

Aptamer-based fluorescence anisotropy (FA)/fluorescence polarization (FP) assays for small molecules recently attract

more attentions as it combines the advantages of aptamers and fluorescence anisotropy [17–20]. FA/FP is related to the rotation of fluorophore during fluorescence lifetime [21]. It is widely used for molecular interaction study and assay developments [21–23]. The FA/FP assay shows merits in simplicity, ease of operation, good precision, high throughput analysis, and high sensitivity [21]. FA assays only need to label one fluorophore on target or affinity ligand. A variety of aptamer-based FA assay formats have been demonstrated for small molecule analysis, e.g., competitive and non-competitive FA assays [16–20, 24–28].

A few groups reported FA analysis of cocaine using aptamer as affinity ligands [16, 26, 28]. Cui et al. developed an aptamer FA assay for cocaine using thrombin as a mass enhancer, based on the aptamer structure switching and thrombin-binding aptamer [16]. Huang et al. reported another FA approach for detection of cocaine by using DNA oligos instead of proteins to generate the FA response caused by cocaine-binding [26]. In one design, the binding of cocaine causes the displacement of one ROX (6-carboxy-x-rhodamine) labeled DNA strand from aptamer, yielding decrease of FA signals. Recently, a sensitive aptamer-based fluorescence polarization assay for cocaine is demonstrated by combining multiple signal amplification processes, such as mass enhancement by proteins, amplification by DNA polymerase, and target recycling through nicking endonuclease [28]. The FP change reaches about 0.250 in the assays [28]. These aptamer FA assays for cocaine involved competition binding or displacement procedures, and all need to use oligonucleotides hybridization or protein-binding to induce FA signal changes, so more reagents or reaction steps are applied in the assays.

We recently have demonstrated a simple direct FA assay for ochratoxin A and ATP only using one tetramethylrhodamine (TMR)-labeled aptamer [20, 25]. This assay relies on the interaction between TMR and the bases (e.g., G bases) of the aptamer, which affects the FA of TMR as such interaction causes fluorescence quenching and influences the local rotation of TMR [20, 29, 30]. The target-binding induces the conformation change of aptamer, altering TMR-G interactions and leading to variance of FA of TMR. This FA strategy is a non-competitive procedure without using DNA oligos, proteins, or nanomaterials to produce large FA change [20, 25, 30].

Herein, we report the development of a direct fluorescence anisotropy assay for cocaine detection by using a TMR-labeled DNA aptamer against cocaine (5'-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC-3') [5, 6, 11, 14], which has a shorter stem at terminals compared with originally reported sequence [11]. We fabricated a series of aptamer probes with a single TMR conjugated on different sites of the cocaine-binding aptamer, including the internal thymine (T) bases in the aptamer and the terminals of the aptamer. By

screening FA responses of each aptamer probe to cocaine, we identified that the aptamer with TMR labeled on the 25th base T showed remarkable FA-decreasing response to cocaine. We achieved a simple, rapid, and sensitive direct FA detection of cocaine using this favorable TMR-labeled aptamer probe. We investigated the effect of experimental conditions (e.g., cations, pH, and temperature) on the performance of the aptamer probe for FA analysis of cocaine. The aptamer probe enabled FA analysis of cocaine in a wide dynamic range of 5 to 5000 μM . Detection of cocaine in diluted serum and urine samples was also achieved, showing the potential of sample analysis in complex sample matrix.

Experimental

Materials and reagents

Cocaine was purchased from the National Institute for Food and Drug Control in China. Caffeine and theophylline were ordered from Aladdin Industrial Corporation. Bovine serum albumin (BSA) and immunoglobulin G (IgG) were purchased from Sigma. L-Arginine, L-phenylalanine, and L-aspartic acid were obtained from Amresco (Solon, OH). All the DNA oligonucleotides were ordered from Sangon Biotech (Shanghai, China). The 30-mer DNA aptamers with a TMR dye labeled at different sites were listed in Table 1, and the labeling position was shown. Other reagents were of analytical grade and obtained from Amresco (Solon, OH) or Beijing Chemical Reagents (Beijing, China). Ultra-pure water from a Purelab Ultra Elga Labwater system was used to prepare solution.

Table 1 List of TMR-labeled DNA aptamers for cocaine

Name	Sequences
C30-5'-TMR	<u>5'</u> (TMR)-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC-3'
C30-12T-TMR	5'-GAC AAG GAA AAT <u>T</u> (TMR) CCT TCA ATG AAG TGG GTC-3'
C30-15T-TMR	5'-GAC AAG GAA AAT CCT <u>T</u> (TMR) TCA ATG AAG TGG GTC-3'
C30-16T-TMR	5'-GAC AAG GAA AAT CCT <u>T</u> (TMR)CA ATG AAG TGG GTC-3'
C30-20T-TMR	5'-GAC AAG GAA AAT CCT TCA AT <u>T</u> (TMR)G AAG TGG GTC-3'
C30-25T-TMR	5'-GAC AAG GAA AAT CCT TCA ATG AAG <u>T</u> (TMR)GG GTC-3'
C30-29T-TMR	5'-GAC AAG GAA AAT CCT TCA ATG AAG TGG GT <u>T</u> (TMR)C-3'
C30-3'-TMR	5'-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC- <u>3'</u> (TMR)

The labeling position of TMR was shown in underline format

Fluorescence measurement

We conducted all of the FA measurements on JASCO FP-8300 fluorescence spectrophotometer (Japan) [20, 25, 30]. The presented procedure was based on our previous experimental procedure [30]. We measured FA of TMR with an excitation at 560 nm and an emission at 578 nm. A total of 5-nm slits was used for the excitation and the emission. For each sample, FA was recorded five times, and the average value was used. Except where noted in the text, we incubated TMR-labeled aptamer (25 nM) with varying concentrations of cocaine in a 200 μ L of buffer solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 0.1% Tween 20 for 20 min, and then FA of TMR was measured in a 100- μ L quartz cuvette at 10 $^{\circ}C$.

Results and discussion

Screening the labeling sites of TMR to construct aptamer probe for FA analysis

Figure 1 shows the principle of direct FA assay for cocaine detection using TMR-labeled aptamer (5'-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC-3') [11]. In a TMR-labeled aptamer for cocaine, TMR may interact with the spatially surrounded G bases of the aptamer, depending on the labeling sites of TMR in the sequence. The TMR-G interaction affects FA values of TMR. Strong interaction causes restricted freedom of TMR and fluorescence quenching, leading to high FA of TMR. Weak interaction allows for a low FA as TMR has more local rotation freedom. The binding of cocaine induces conformation change of aptamer [11], then possibly alters the TMR-G interactions and the local environment of TMR. As a consequence, FA of TMR changes. Thus,

cocaine can be detected by monitoring FA. A key for the success of this strategy is to find a suitable TMR-labeling position that allows the TMR-labeled aptamer to show binding-induced FA responses to cocaine with good sensitivity.

We conjugated single TMR on different sites of the 30-mer DNA aptamer against cocaine to screen a favorable labeling position of TMR. The screened labeling sites included 3' terminal, 5' terminal, and internal thymine (T) bases of the aptamer (12T, 15T, 16T, 20T, 25T, and 29T) (shown in Table 1). We measured the FA responses of each TMR-labeled aptamer (25 nM) to cocaine in the binding buffer solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 0.1% Tween 20 at 10 $^{\circ}C$. In the absence of cocaine, all TMR-labeled aptamers showed FA values higher than 0.210 (Fig. 2a). Figure 2b shows the FA changes of each TMR-labeled aptamer in the presence of cocaine at 1 mM. These TMR-labeled aptamer probes showed distinct FA responses to cocaine. C30-29T-TMR and C30-3'-TMR gave negligible FA responses to cocaine. The rest of TMR-labeled aptamer probes all exhibited FA-decreasing responses to cocaine. Among these tested aptamer probes, C30-25T-TMR exhibited largest FA changes upon addition of cocaine. Two control oligonucleotides (5'-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3', 5'-CCT GGG GGA GTA TTG CGG AGG AAG G-3') with TMR labeled on 5' end both did not show FA response to cocaine, showing the aptamer binding to cocaine is sequence dependent. In addition, we can identify the change in fluorescence anisotropy of TMR-labeled aptamer is not caused by the changes in complex molecular mass because relative to the molecular mass of TMR-labeled aptamer (about 9.3–9.8 kDa), the increased mass caused by cocaine (about 0.34 kDa) is not large. Therefore, we chose C30-25T-TMR as the favorable aptamer probe to detect cocaine in the FA assays.

Fig. 1 Principle of direct fluorescence anisotropy assay for cocaine using a TMR-labeled aptamer probe. The binding of cocaine to the TMR-labeled aptamer causes conformation change and alteration of the intramolecular interaction between TMR and bases of aptamer, leading to variance of FA of TMR. The direct measurement of FA allows for the detection of cocaine

Fig. 2 (a) FA values of TMR-labeled aptamers in absence of cocaine. (b) FA changes of TMR-labeled aptamer upon cocaine (1 mM). The binding buffer solution consisted of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , and 0.1% Tween 20. Incubation temperature was 10 °C

Optimization of FA assay condition

To get the optimum experimental conditions for sensitive FA assay, we investigated the influence of experimental conditions on FA responses of C30-25T-TMR to cocaine, including buffer composition, buffer pH, and temperature by monitoring the FA changes of C30-25T-TMR (25 nM) caused by the presence of cocaine (1 mM) in different conditions.

MgCl_2 in binding buffer solution had large effect on FA signal change. As shown in Fig. 3, when cocaine was not present, the FA of C30-25T-TMR first increased as MgCl_2 concentration increases and then gradually decreased when MgCl_2 concentration was higher than 2 mM. When cocaine was added, the FA of C30-25T-TMR significantly decreased even if the binding buffer solution did not contain MgCl_2 . The addition of MgCl_2 could lead to larger FA change of C30-25T-TMR caused by cocaine. As shown in Fig. 3b, the absolute FA change increased with the increase of concentration of MgCl_2 , and the maximum absolute FA change of C30-25T-TMR caused by cocaine was obtained with 2 mM MgCl_2 . The higher concentration of MgCl_2 could lead to the decrease of the absolute FA change. The possible reason for this phenomenon is that Mg^{2+} at optimal concentrations is desired for

better binding between the aptamer and cocaine, which may help the aptamer to keep a good conformation for affinity binding. Therefore, MgCl_2 concentration was fixed at 2 mM for subsequent experiments.

Then, we assessed the influences of NaCl concentration in the binding buffer solution on the FA response of C30-25T-TMR upon cocaine (Fig. S1A in Electronic Supplementary Material (ESM)). As shown in Fig. S1B, the largest absolute FA change of C30-25T-TMR was observed when 50 mM NaCl existed (Fig. S1B in ESM). The reason for this phenomenon may be the simultaneous presence of divalent cations and monovalent ions at optimal concentrations is needed for maintaining good conformation and binding affinity of the aptamer to generate FA response. Thus, the addition of 50 mM NaCl in the binding buffer solution was applied in the further experiments.

The concentration of KCl in the binding buffer solution also exhibited influence on FA response of C30-25T-TMR to cocaine. Fig. S2A in ESM shows the FA of C30-25T-TMR was relatively low in the blank sample without containing cocaine when KCl was absent, and the FA of C30-25T-TMR in the blank sample increased sharply after the addition

Fig. 3 (a) Effects of MgCl_2 on the FA of C30-25T-TMR in the absence or the presence of cocaine (1 mM) in the binding buffer solution containing 50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 5 mM KCl, 0.1% Tween 20, and various concentrations of MgCl_2 at 20 °C. (b) The FA change of C30-25T-TMR caused by cocaine vs MgCl_2 concentration

Fig. 4 (a) The influence of pH of binding buffer solution on the FA of C30-25T-TMR in the absence of or in the presence of cocaine (1 mM). (b) The absolute FA change caused by cocaine vs pH of binding buffer solution. The binding buffer solution containing 50 mM Tris-HCl, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, and 0.1% Tween 20. Incubation temperature was 20 °C

of KCl. It shows that KCl may change the conformation of aptamer and the surrounded environment of labeled TMR. In the presence of cocaine, decreased FA of C30-25T-TMR was observed in the tested concentration range of KCl. The absolute FA change of C30-25T-TMR caused by cocaine gradually increased with the addition of KCl (Fig. S2B in ESM). When KCl concentration was higher than 5 mM in the binding buffer solution, the absolute FA change reached the maximum level. Hence, the KCl concentration was fixed at 5 mM in the binding buffer solution for subsequent experiments.

We also examined the influence of pH on the affinity binding of aptamer by FA analysis (Fig. 4). When the pH of the binding buffer solution was lower than 5.0, only small absolute FA change caused by cocaine was observed (shown in Fig. 4b). The low pH possibly weakens the binding affinity of the aptamer. Large FA changes were obtained in the pH range from 7.0 to 8.5. Binding buffer solution at pH 7.5 was chosen in our FA analysis as the maximum cocaine-induced FA change was achieved, and this pH is also close to the physiological condition. Finally, the binding buffer solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, and 0.1% Tween 20 were applied.

Temperature has a great effect on the FA analysis using aptamers, which not only influences the rotation of aptamer

probe but also affects the binding affinity of aptamer. As shown in Fig. 5a, the FA of C30-25T-TMR in the absence of cocaine decreased with the temperature increase, which can be explained by the increase of the rotation of the labeled TMR with increasing temperature. In the presence of cocaine (Fig. 5b), the obtained absolute FA change of C30-25T-TMR caused by cocaine was large when the temperature was lower than 10 °C. When the temperature was higher than 10 °C, the absolute FA change of C30-25T-TMR caused by cocaine becomes decreased with the increase of temperature. High temperature may affect the conformation of aptamer, properties of TMR, and FA of TMR in affinity complex. FA response of C30-25T-TMR to cocaine completely disappeared at 40 °C, which is probably due to the loss of the binding affinity of aptamer as the high temperature can disrupt the structure of aptamer. Thus, in the following experiments, FA analysis of cocaine at 10 °C, instead of 20 °C, was applied to achieve better sensitivity in the assays.

FA analysis of cocaine

At optimized experimental conditions, we successfully detected cocaine with C30-25T-TMR by FA analysis. With the

Fig. 5 (a) Effects of temperature on the FA of C30-25T-TMR in the absence or the presence of cocaine in the binding buffer solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, and 0.1% Tween 20. (b) The relationship between the FA change caused by cocaine and the temperature

increase of cocaine, the FA of C30-25T-TMR gradually decreased. The dynamic detection range was from 5 to 5000 μM (Fig. 6a) ($y = -0.088 \times / (82.9 + x) + 0.262$, $R^2 = 0.999$), covering about three orders of magnitude, and the detection limit of cocaine was determined to be 5 μM . The relative standard deviations for five repeated analyses were less than 3%. As comparison, the FA changes of other TMR-labeled aptamer probes upon addition of cocaine are shown in Fig. 6b. It confirms that C30-25T-TMR shows the best performance in FA analysis among the different TMR-labeled aptamer probes. In addition, the fluorescence intensity of C30-25T-TMR increased with increasing concentrations of cocaine, and the maximum fluorescence intensity increase was about 73%. It suggests that the interaction between G base and TMR is weakened upon cocaine since the TMR-G interaction can cause fluorescence quenching of TMR, which is in agreement with previous studies [29]. The binding of cocaine reduced TMR-G interaction, reduced fluorescence quenching, and increased the local rotation of TMR, so the FA of C30-25T-

TMR decreased with the increase of cocaine concentration. However, the detail of how TMR interacts with which adjacent G remains unknown. Compared with the reported aptamer-based FA methods for cocaine detection [16, 26, 28], our method is simpler as we only need one TMR-labeled aptamer. The reported aptamer-based FA methods need to use protein or oligonucleotide to help trigger FA signal change, relying on the target-binding that competes with the hybridization of complementary oligonucleotides [16, 26, 28]. In addition, the maximum FA change in the previous report was small, only about 0.010 in FA changes [16, 26]. In our method, the maximum FA change was more than 0.080. The detection limit of our method is comparable to that previously reported [16, 26]. The signal amplification-based FA method has achieved higher sensitivity than that of our methods for detection of cocaine [28]. The sensitivity of our method is also comparable to that from colorimetric assay using unlabeled aptamer based on binding-induced displacement of bound dye [14] and that reported from aptamer-based electrochemical sensor [11].

Fig. 6 (a) Fluorescence anisotropy of C30-25T-TMR in response to varying concentrations of cocaine. (b) Fluorescence anisotropy changes of different TMR-labeled aptamers in response to varying concentrations of cocaine

Assay selectivity and performance in complex sample matrix

We tested the selectivity of the FA assays for cocaine. Some components including theophylline, caffeine, BSA, IgG, and a few amino acids including arginine, phenylalanine, and aspartic acid were tested along with cocaine. In Fig. 7, no remarkable FA changes of C30-25T-TMR were induced by the tested molecules at relatively high concentrations. When cocaine and the tested molecules both existed in the sample

Fig. 7 Selectivity of the aptamer probe C30-25T-TMR for cocaine analysis. The FA responses of C30-25T-TMR were tested in the absence of cocaine (a) or in the presence of 1 mM cocaine (b), 10 mM arginine (c), 2 mM phenylalanine (d), 2 mM aspartic acid (e), 100 μM BSA (f), 20 μM IgG (g), 200 μM theophylline (h), and 100 μM caffeine (i). The FA responses of C30-25T-TMR were also tested in the simultaneous presence of 1 mM cocaine and various compounds like 10 mM of arginine (j), 2 mM phenylalanine (k), 2 mM aspartic acid (l), 100 μM BSA (m), 20 μM IgG (n), 200 μM theophylline (o), or 100 μM caffeine (p)

mixture, the FA caused by cocaine was not greatly affected by the tested molecules. It indicated that tested molecules did not interfere with the detection of cocaine.

We further tested the feasibility of FA assay for detection of cocaine in complex sample matrix (diluted serum sample and urine sample). Cocaine was spiked in 100-fold diluted, or 20-fold diluted, human serum and then were analyzed by using C30-25T-TMR (Fig. S3 in ESM). The obtained FA change became relatively smaller in diluted human serum samples. In 20-fold diluted serum sample, the detection limit was 20 μM . The presence of components in serum samples has some effects on the detection of cocaine. Some proteins existing in serum sample may non-specifically bind with the DNA oligonucleotides. In addition, cocaine may interact with some abundant proteins in serum sample. In 100-fold diluted serum sample, cocaine at 10 μM was still detected with a higher sensitivity than that in 20-fold diluted serum sample. C30-25T-TMR also showed FA good response upon cocaine in 10-fold diluted urine sample, and the detection limit of cocaine was 5 μM . These results show the C30-25T-TMR aptamer probe can be applied to detect cocaine in complex sample matrix.

Conclusion

In summary, we developed a simple direct fluorescence anisotropy assay for cocaine using TMR-labeled anti-cocaine aptamer. This method is based on binding-induced alteration of the intramolecular interaction between TMR and adjacent guanine bases of aptamer and FA changes of labeled TMR. Among the aptamers with single TMR labeled on terminal or internal thymine bases, we identified the aptamer with TMR labeled on 25th T in the sequence showed remarkable FA-decreasing responses to cocaine. This aptamer probe allowed for detection of cocaine with large FA changes, enabling the detection of cocaine at 5 μM . The aptamer probe also allowed for the detection of cocaine in diluted serum sample and urine sample. This work provides an easy and fast method for direct FA detection of cocaine using TMR-labeled aptamers, showing advantages in simplicity and sensitivity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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