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Amplified Detection of Cocaine Based on Strand-Displacement Polymerization and Fluorescence Resonance Energy Transfer

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Abstract

Cocaine is one of the most abused drugs in the United States, and is potentially dangerous when consumed in excess. Its detection is thus important in many areas in the fight against drug trafficking. We have developed an amplified detection method for cocaine based on a strand-displacement polymerization reaction using aptamer recognition. The system mainly consists of a hairpin probe with Cy5 labeled on its 3' end, a primer with FAM labeled on its 5' end, and polymerase. The aptamer sequence is integrated into the 5'-section of the hairpin probe. The primer is designed complementary to the 3' end of the hairpin probe, which is also part of the hairpin stem region. The cocaine induced reaction cycle generates product for detection and thus for signal amplification. The detection limit of this method is 200 nM in about 16 min and the specificity of this approach is excellent. We believe that this strategy will be useful for the development of analytical schemes for a variety of aptamers for small molecules, metal ions, and proteins. This simple scheme employing the strand-displacement polymerization reaction may find wide application in forensic analysis, environmental monitoring, and clinical diagnostics.

Keywords

Cocaine; Detection; Aptamer; Fluorescence resonance energy transfer; Strand-displacement polymerization

1. Introduction

In the United States, cocaine is classified as an illegal hard drug, making its detection important at all levels of law enforcement, as well as in clinical diagnosis. New methods of cocaine detection have emerged based on the advancement of analytical technologies employing aptamers (Stojanovic et al., 2000; Stojanovic et al., 2001). Aptamers are single-stranded oligonucleotides with specific recognition properties towards a great variety of target molecules or cells, and they are generated by an *in vitro* selection process called

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SELEX (selective evolution of ligands by exponential enrichment) (Ellington et al., 1990; Tuerk et al., 1990; Shangguan et al., 2006). Previously, aptamer-based cocaine assays have made use of colorimetric (Stojanovic et al., 2002; Liu et al., 2006; Zhang et al., 2008; Zhu et al., 2010), electrochemical (Baker et al., 2006; Du et al., 2010), and fluorescence (Stojanovic et al., 2000; Stojanovic et al., 2001; Wu et al., 2010) methods for quantification. However, the generally low affinity of the cocaine aptamer, having a K_d of only 5 μM , results in relatively high detection limits for these reported aptameric assays (Stojanovic et al., 2000; Stojanovic et al., 2001). To address this problem, several aptamer-based signal amplification strategies have been developed, involving enzyme conjugates (Freeman et al., 2009), DNazymes (Elbaz et al., 2009), quantum dots (Zhang et al., 2009), magnetic beads (Yan et al., 2010), and the DNA machine (Shlyahovsky et al., 2007). Recently, our laboratory reported use of a strand-displacement polymerization reaction on a hairpin fluorescence probe to detect DNA fragments with high sensitivity. The target DNA opens the hairpin fluorescence probe and triggers a circular strand-displacement polymerization reaction. One target DNA can open many hairpin fluorescence probes, increasing detection sensitivity by nearly five orders of magnitude (Guo et al., 2009). Subsequently this amplification technique combined with an aptamer was used for target detection (He et al., 2010). However, the aptamer needed to be split, and the duplex intercalating fluorescent dye resulted in a rather high background. In addition, the single fluorescence signal was affected by environmental and instrumental factors. A method based on fluorescence ratio measurement can minimize these effects and enable more precise detection.

Herein, a strand-displacement polymerization reaction is used to amplify the recognition event between the aptamer and its cocaine target, with signal production by fluorescence resonance energy transfer (FRET). In this system, the cocaine aptamer is integrated into the hairpin probe, with a donor fluorophore (FAM) and an acceptor fluorophore (Cy5) tethered to the ends of the primer and hairpin probe, respectively (Table S1). After the aptamer/substrate binding event opens the hairpin probe, the primer binds to the exposed stem of the hairpin probe. This brings the donor and acceptor into close proximity, which quenches the donor fluorescence and, at the same time, enhances the acceptor signal. Using this system, a detection limit of 200 nM cocaine can be achieved within a 16-min reaction period.

2. Experimental

2.1. Chemicals and reagents

The sequences of DNA oligonucleotides are listed in Table S1. The hairpin probe with Cy5 label at the 3' end was purchased from Integrated DNA Technologies (IDT). Primers labeled with FAM at the 5' end were synthesized with standard phosphoramidite chemistry. All DNA synthesis reagents were purchased from Glen Research. All reagents for HPLC purification and gel electrophoresis were from Fisher Scientific. Cocaine was from Sigma. Polymerase Klonow Fragment (3'-5' exo-), Buffer (50mM NaCl, 10mM Tris-Cl, 10mM MgCl_2 , 1mM Dithiothreitol (DTT), pH 7.9) and mixed dNTPs were from New England Biolabs (NEB).

2.2. Apparatus and measurements

DNA synthesis was performed on an ABI 3400 DNA/RNA synthesizer (Applied Biosystems). A Prostar HPLC (Varian) with C18 reverse-phase column (Alltech, 5 μm , 250 $\times$ 4.6mm) was used for probe purification. Absorbances were measured on a Cary-Bio300 UV/vis spectrometer (Varian) and used to calculate probe concentrations. Fluorescence data were obtained using a Fluorolog-3 spectrofluorometer (Jobin Yvon) with the temperature controller set to 37 $^\circ\text{C}$. The excitation wavelength was 488 nm, and emission wavelengths were 512nm (FAM) and 655nm (Cy5), with 5 nm bandwidths. When the fluorescence

intensity became steady, the target was added to the mixture, and the fluorescence intensity was recorded as a function of time. The titration curve was used to estimate the detection limit of the method based on 3 times the standard deviation of 5 measurements of blank samples.

For gel experiments, all samples were incubated at 37 °C in the NEB buffer for about 1 hour before use. The 15% native polyacrylamide gel was prepared using 1 × TBE buffer (89 mM Tris Borate, 2.0 mM Na₂EDTA, pH 8.3). The gel was run at 100 V for 60 min in 1 × TBE buffer, stained for 30 min in a solution (500 ml formamide, 100 ml 10 × TBE, 400 ml H₂O, 100–200 mg Stains All), illuminated with white light until the stained band appeared, and photographed with a digital camera.

3. Results and discussion

3.1. Principle of the amplified cocaine detection method

Scheme 1 outlines the working principle of this strand-displacement polymerization reaction. Briefly, the system consists of a hairpin probe with Cy5 labeled on its 3'-end, a primer with FAM labeled on its 5'-end, and polymerase. The aptamer sequence is integrated into the 5'-end of the hairpin probe, as shown in Table S1. The primer is designed to be complementary to the 3'-end of the hairpin probe, which is also part of the hairpin stem region. In the absence of target, the 3'-end of the probe is unable to bind with the primer, and the Cy5 label does not fluoresce (state 1). However, in the presence of the cocaine target, the hairpin probe recognizes and binds to it and undergoes a conformational change, leading to stem separation (state 2). Following this, the primer anneals with the open 3'-end and triggers a polymerization reaction in the presence of dNTPs/polymerase (state 3). At the same time, FAM is brought into close proximity with Cy5, resulting in fluorescence resonance energy transfer. In the process of primer extension, the target is displaced by the polymerase with strand-displacement activity, after which a complementary DNA is synthesized, forming a probe–cDNA complex (state 4). Finally, to renew the cycle, the displaced target binds to another hairpin probe, which triggers another polymerization reaction. Throughout this cyclical process, additional Cy5 and FAM are brought together, and the FRET signal increases. Thus, sensitive detection of cocaine is achieved by monitoring the fluorescence intensities of Cy5 (acceptor) and/or FAM (donor).

3.2. Design of the hairpin probe and primer

The probe was designed using the cocaine aptamer as a recognition module for the sensitive detection of cocaine. Seven bases of the cocaine aptamer (38 nt) are incorporated into the stem. The overall length of the stem (10bp) is sufficient to maintain the stem-loop structure in the absence of cocaine. To guarantee that the length of primer is optimum in this method, primers of four different lengths were synthesized and tested, as shown in Table 1. The results show that primer (8mer) provides optimal response (Fig. S1). Finally, FRET was chosen as a signal to monitor the cyclical process, with FAM and Cy5 as the donor/acceptor pair (Yang et al., 2006). When excited at 488 nm, the primer emits strongly around 515 nm (FAM), in the absence of cocaine, with negligible Cy5 emission at 655 nm. Upon addition of the target, fluorescence of FAM decreases and emission of Cy5 increases.

3.3. Verification of the amplified cocaine detection method

To prove the principle of the scheme, the real-time FRET signal was monitored. As shown in Fig. S2, both the hairpin probe and primer contribute to the fluorescence background, but pure polymerase and pure cocaine have no impact on the FRET signal. Fig. S2 (A) shows that there is no FRET signal with primer (8mer), hairpin probe, dNTPs, and polymerase present (no false positive), and that FRET occurs when cocaine is added. Likewise, Fig. S2

(B) shows that there is no FRET signal until polymerase is added. This means that the 8-nt primer does not bind to the hairpin probe even in the presence of cocaine, because the 8-bp duplex is not sufficiently stable at 37 °C. However, in the presence of polymerase, the duplex is extended and stable at this temperature. Electrophoresis of the products of the polymerization reaction provides direct evidence of the binding principle. As shown in Fig. S3, the new band in lane 4 likely corresponds to the probe–cDNA complex (state 4 in the scheme), which is synthesized only when all five assay components (primer (8mer), hairpin probe, dNTPs, polymerase and cocaine) are present. In order to further confirm that the method can quantify the concentration of cocaine, the emission fluorescence intensity of FAM and Cy5 were monitored simultaneously (Fig. S4). Here the initial point is normalized to 1, and, with time, the Cy5 signal increases above 1 and the FAM signal decreases below 1 as the concentration of cocaine increases.

3.4. Amplified detection of cocaine with high sensitivity

The FRET signal is defined as the fluorescence intensity ratio $Cy5/FAM (E_{m655\text{ nm}}/E_{m515\text{ nm}})$. Figure 1 shows the FRET signal as a function of time with different concentrations of target molecule cocaine. The results showed that the FRET signal increased as the cocaine concentration increased. In order to quantitate the relationship between signal and cocaine concentration, we defined the fluorescence ratio enhancement (1000s) as follows:

$$\text{Fluorescence ratio enhancement (1000s)} = F_{1000} - F_0$$

Here the background (F_0) is defined as $Cy5/FAM (0s)$, and the signal (F_{1000}) is taken as $Cy5/FAM (1000s)$. Fig. S5 shows the relationship between the Fluorescence ratio enhancement (1000s) and the concentration of cocaine. The fluorescence ratio enhancement (1000s) increased significantly with an increase of cocaine concentration until a plateau was reached. A good linear relationship (correlation coefficient = 0.982) was observed between the square root of the fluorescence ratio enhancement (1000s) and the logarithm of the cocaine concentration from 200 nM to 200 μM , as shown in Fig. S6. For this method, the detection limit is defined as the concentration of target that yields a net signal (total signal minus background) equivalent to three times the standard deviation of a series of replicates of background (Li et al. 2008). The standard deviation of F_0 in this study is 0.03 ($3 \times 0.03 = 0.09$), corresponding to a concentration of 0.2 μM . Therefore, 0.2 μM is considered to be the detection limit of this method.

3.5. Selectivity of the amplified cocaine detection method

To verify the selectivity of the amplified cocaine detection method, benzoylecgonine (BE) and ecgonine methyl ester (EME) were chosen as negative controls. The two molecules are cocaine metabolites which have been reported to have no affinity for the cocaine aptamer (Stojanovic et al., 2000; Stojanovic et al., 2001; Zhu et al., 2010). As shown in Figure 2, our results indicated that the method is highly selective for its target cocaine compared to its metabolites BE and EME. It has been noted that this cocaine aptamer sequence has been found to bind with steroids (Stojanovic et al., 2003) and quinine (Taylor et al., 2009). Thus, it would be very desirable to have a more specific cocaine aptamer.

4. Conclusions

In conclusion, an amplified detection method for cocaine based on a strand-displacement polymerization reaction and FRET has been developed. Binding of the target cocaine to the aptamer causes the opening of the hairpin probe, triggering the strand-displacement

polymerization reaction to cycle the entire reaction. The detection limit of this method is 200 nM reached in about 16 min, and the response to cocaine analogs is very small. Unlike other aptamer sensing platforms based on thermodynamic equilibrium, the proposed method is based on kinetics, which could easily be used for real-time monitoring of the entire reaction. Presumably, with advances in the development of a variety of aptamers for small molecules, metal ions, and proteins, this strand-displacement polymerization reaction strategy may find wide application in forensic analysis, environmental monitoring, and clinical diagnostics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.

Working principle of amplified detection of cocaine by strand-displacement polymerization reaction and FRET.

Figure 1.

Fluorescence intensity ratio of Cy5/FAM (Em_{655nm}/Em_{515nm}) time response for different concentrations of cocaine. The curves from top to bottom contain cocaine at concentrations of 5×10^{-3} , 1×10^{-3} , 2×10^{-4} , 4×10^{-5} , 8×10^{-6} , 1.6×10^{-6} , 3.2×10^{-7} and 0M, respectively. The data for the figure have been normalized. All samples were incubated at 37 °C and contained 100 μ L of solution consisting of 100 nM probe and 100 nM primer (8mer), 25 U polymerase, 100 μ M dNTPs, 1 $\times$ NEB buffer.

Figure 2. Fluorescence intensity ratio Cy5/FAM at 1000s for cocaine, benzoylecgonine (BE) and ecgonine methyl ester (EME) (5 mM each).