Published in final edited form as:

Anal Chem. 2018 February 06; 90(3): 1748–1758. doi:10.1021/acs.analchem.7b03625.

Sensitive Detection of Small-Molecule Targets Using Cooperative Binding Split Aptamers and Enzyme-Assisted Target Recycling

Haixiang Yu, Juan Canoura, Bhargav Guntupalli, Obtin Alkhamis, and Yi Xiao*

Department of Chemistry and Biochemistry, Florida International University, 11200 SW Eighth Street, Miami, Florida 33199, United States

Abstract

Signal amplification via enzyme-assisted target recycling (EATR) offers a powerful means for improving the sensitivity of DNA detection assays, but it has proven challenging to employ EATR with aptamer-based assays for small-molecule detection due to insensitive target response of aptamers. Here, we describe a general approach for the development of rapid and sensitive EATR-amplified small-molecule sensors based on cooperative binding split aptamers (CBSAs). CBSAs contain two target-binding domains and exhibit enhanced target response compared with single-domain split aptamers. We introduced a duplexed C3 spacer abasic site between the two binding domains, enabling EATR signal amplification through exonuclease III's apurinic endonuclease activity. As a demonstration, we engineered a CBSA-based EATR-amplified fluorescence assay to detect dehydroisoandrosterone-3-sulfate. This assay achieved 100-fold enhanced target sensitivity relative to a non-EATR-based assay, with a detection limit of 1 μ M in 50% urine. We further developed an instrument-free colorimetric assay employing EATR-mediated aggregation of CBSA-modified gold nanoparticles for the visual detection of low-micromolar concentrations of cocaine. On the basis of the generalizability of CBSA engineering and the robust performance of EATR in complex samples, we believe that such assays should prove valuable for detecting small-molecule targets in diverse fields.

Graphical abstract

*Corresponding Author: yxiao2@fiu.edu.

ORCID

Yi Xiao: 0000-0001-7278-9811

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.7b03625. DNA sequences, ITC data, fluorescence binding curve of DIS–CBSA-4536, digestion time-course of CBSAs, CBSA-based detection of DIS in 50% urine, time-courses of digestion for CBSAs with different short fragment variants, characterization of DNA surface coverage on CBSA short-fragment-modified AuNPs, time dependence of Exo III digestion of AuNPs displaying CBSA short fragments at various surface coverages, UV–vis absorbance measurements of colorimetric cocaine assay, schematic of modified CBSA cocaine assay, and time dependence of Exo III digestion with cocaine CBSA assays (PDF)

The biosensor field is on a continuous quest for ever-greater sensitivity. In conventional bioassays, where the signal is directly proportional to the target concentration, the sensitivity is determined by the intrinsic target affinity of the bioreceptor being used for detection.¹ In this scenario, it would be difficult to generate a measurable signal at target concentrations more than 100-fold lower than the dissociation constant (K_D) of the bioreceptor.

Accordingly, many amplification approaches have been developed in which one binding event can generate multiple signals,^{2,3} allowing detection of targets at very low concentrations. Enzyme-assisted target recycling (EATR) has proven to be an especially effective way to amplify signals generated from target-binding events.⁴ This approach relies on selective, nuclease-mediated degradation of the probe strand of a target–probe duplex that only forms in the presence of target; this liberates the target, which is “recycled” for use in additional digestion reactions. For example, EATR has been used with molecular beacon probes for DNA detection.³ The molecular beacon probe contains a loop sequence complementary to the target DNA, with a fluorophore–quencher pair attached to the stem of the beacon as a signal-reporting element. When the molecular beacon probe binds the target DNA, it switches from a stem–loop structure into a probe–target duplex that contains a structural⁵ or sequence³ recognition site for a nuclease. This enzyme then catalyzes the digestion or cleavage of the beacon probe, liberating the fluorophore and releasing the target, which can then hybridize with another molecular beacon probe. Thus, a substantial fluorescent signal can be generated by a single copy of the target through EATR, greatly decreasing the limit of detection.^{3,5} Given that DNA can be easily modified with various signaling reporters^{3,6–8} and that different nucleases can be utilized to process the target–probe duplex,^{3,5,7,9} many EATR-based assays have been developed for DNA detection with ultrahigh sensitivity.⁴

Aptamers are nucleic acid-based biorecognition elements that are isolated *in vitro* through processes based on systematic evolution of ligands by exponential enrichment (SELEX).¹⁰ Aptamers can bind to a wide variety of targets, including proteins, metal ions, small molecules, and even whole cells,¹¹ and aptamer-based biosensors have been developed for environmental monitoring, drug detection, and medical diagnostics.^{12–14} Aptamer-based sensors have many advantages compared to other state-of-the-art methods for the detection of small-molecule targets. Instrumental methods, such as gas or liquid chromatography/mass spectrometry, are sensitive and specific. However, these methods require laborious sample preparation and cumbersome and sophisticated instruments, limiting their use for on-site and high-throughput detection.¹⁵ Antibody-based immunoassays, such as enzyme-linked immunosorbent assay (ELISA), are highly sensitive and offer high target specificity. However, the *in vivo* processes for antibody generation are tedious, costly, and challenging

for non-immunogenic small molecules.¹⁶ On the other hand, aptamers can be isolated rapidly with controllable affinity and specificity, and are produced without batch-to-batch variation.¹⁶ Many strategies have been employed into aptamer-based assays to achieve target detection in an instrument-free manner.¹⁷ In particular, aptamers can be split into two or three fragments that remain separate in the absence of target but assemble upon target binding,^{18,19} and such split-aptamer-based sensors have gained popularity as a potential strategy for effective signal reporting.²⁰ In principle, the target-induced assembly of split aptamers should be compatible with EATR-mediated signal amplification. Ideally, the split fragments should be highly responsive to target-induced assembly and only the target-aptamer complex should be specifically recognized and digested by the enzyme. However, it is difficult to achieve sensitive target-induced aptamer assembly with conventional split aptamers with a single binding-domain. Small-molecule-binding split aptamers usually have equilibrium dissociation constants (K_D) in the high-micromolar range, such that no measurable target-induced aptamer assembly can be observed even with a high concentration of targets.^{21,22} Although target affinity can be improved by engineering split aptamers with longer complementary stems, the majority of thermostable split aptamers undergo some degree of preassembly in the absence of target,²² producing high background signal. Second, in contrast to DNA-based EATR assays, in which target-binding always converts a stem-loop probe structure into a probe-target duplex, aptamer-target binding often gives rise to complex tertiary structures that are nonideal substrates for nucleases. For example, three-way junctions and G-quadruplexes have been shown to inhibit exonuclease I,²³ flap endonuclease I,²⁴ and polymerases.²⁵ Additionally, target binding within the aptamer can interfere with enzyme binding and activity; for example, some small-molecule DNA-intercalating agents and DNA adducts can inhibit nucleases, especially exonucleases.^{24,26,27}

To overcome these limitations, we have developed a strategy for generating cooperative binding split aptamers (CBSAs) for use in EATR-amplified biosensors. CBSAs are engineered from parent split aptamers with a single target-binding domain and consist of a short fragment and a long fragment that contain two target-binding domains. The first binding event partially stabilizes the CBSA structure such that the second binding event can occur. This cooperative assembly can reduce the concentration change required to assemble the CBSA severalfold, resulting in far greater specificity and sensitivity relative to the parent split aptamer.^{22,28} For example, our group has previously demonstrated the successful engineering of a highly target-responsive CBSA for cocaine, which enables specific, ultrasensitive, one-step fluorescence detection of cocaine within 15 min in 10% saliva.²² Here, we have developed a CBSA-based EATR-amplified fluorescence assay for the detection of dehydroisoandrosterone-3-sulfate (DIS) in urine samples. We modified our short fragment to incorporate a C3 spacer abasic site;²⁹ when the CBSA assembles, the resulting duplex at the abasic site is recognized and cleaved by exonuclease III (Exo III). This results in recycling of the long fragment and DIS molecules for further rounds of digestion, while releasing a fluorescent signal for each digestion event. We demonstrated that this assay exhibits 100-fold enhanced sensitivity relative to the CBSA alone without EATR signal amplification. Importantly, this assay also proved highly sensitive when used in urine samples, allowing us to detect DIS in 50% urine within 30 min at concentrations as

low as 1 μM . To demonstrate the generality of our approach for the detection of other small-molecule targets, we also developed an assay that utilizes EATR-mediated aggregation of CBSA fragment-modified gold nanoparticles (AuNPs) for colorimetric detection of cocaine. This assay was able to successfully achieve naked-eye detection of low-micromolar concentrations of cocaine within 20 min. We believe that our approach should offer a general strategy for the development of other CBSA-based, EATR-amplified assays for the rapid, sensitive, and specific detection of various small-molecule targets in complex samples in both clinical and field settings.

EXPERIMENTAL SECTION

Materials

All DNA strands used in this work were synthesized by Integrated DNA Technologies and purified with high-performance liquid chromatography (HPLC). DNA was dissolved in PCR grade water, and DNA concentrations were measured with a NanoDrop 2000 (Thermo Scientific). The sequences of the oligonucleotide strands are listed in the Supporting Information (Table S1). Tris(hydroxymethyl)-aminomethane (Tris), hydrochloric acid, sodium chloride, magnesium chloride, bovine serum albumin (BSA), 1,4-dithiothreitol (DTT), gold(III) chloride trihydrate, cocaine hydrochloride, and DIS were purchased from Sigma-Aldrich. Exo III was purchased from New England Biolabs. OliGreen and SYBR gold were purchased from Thermo Fisher Scientific. Urine samples used in this work were collected from healthy and consenting adult donors.

Isothermal Titration Calorimetry (ITC)

ITC experiment was performed with a MicroCal ITC200 (Malvern). A solution of 0.2 mM DIS was titrated into 10 μM DISS.1-AT, with both diluted in DIS binding buffer (10 mM Tris-HCl, 0.5 mM MgCl_2 , pH 7.4). The experiment consisted of 19 successive 2 μL injections after a 0.4 μL purge injection with spacing of 180 s at 23 $^{\circ}\text{C}$. The injection heat was analyzed with the MicroCal analysis kit integrated into Origin 7 software, and the titration curve was fitted with a single-site binding model.

Determination of Binding Affinity of DIS-CBSA-4536, DIS-SA-536, and DIS-SA-36

The binding affinities of the split aptamers were determined fluorescently as reported previously²² with some modifications. Amounts of 1 μL of 100 μM DIS long fragment (DIS-LF, DIS-LF-536 or DIS-LF-36), 1 μL of 100 μM respective fluorophore/quencher-modified DIS short fragment (FQ-DIS-SF, FQ-DIS-SF-536, mixed with 5 μL of solution containing various concentrations of DIS. An amount of 80 μL of each sample was then transferred into wells of a 96-well plate. After a 30 min incubation at room temperature, the fluorescence was measured using a TECAN M1000 Pro ($\lambda_{\text{ex/em}}$ = 648/668 nm). Each sample was analyzed in triplicate, and the means and standard deviations were plotted. The data were fitted with the Hill equation using Origin 9 to calculate Hill coefficient (n_{H}) and $K_{1/2}$ (DIS concentration producing half occupancy) of each split aptamer.

Exo III Digestion and Gel Electrophoresis Analysis

All digestion experiments were performed as followed unless specified otherwise. Amounts of 1 μL each of 50 μM short and long CBSA fragments were added into 43 μL of reaction buffer to yield a final DIS and cocaine concentrations of 250 and 500 μM , respectively. The buffer composition varied for each aptamer/target composition, with final concentrations for each reaction as follows: DIS (10 mM Tris, 0.5 mM MgCl_2 , 0.1 mg/mL BSA, pH 7.4) and cocaine (10 mM Tris, 0.1 mM MgCl_2 , 0.1 mg/mL BSA, pH 7.4). After incubation at 23 $^{\circ}\text{C}$ for 30 min, 5 μL of 0.01 U/ μL Exo III was added. After 5, 10, 15, 30, and 60 min of digestion, 5 μL of each sample was collected and immediately mixed with 10 μL of loading buffer (71.25% formamide, 10% glycerol, 0.125% SDS, 25 mM EDTA, and 0.15% (w/v) xylene cyanol) to inactivate Exo III. Control samples were prepared similarly, but with 5 μL of reaction buffer instead of Exo III. The digestion products were characterized via denaturing polyacrylamide gel electrophoresis (PAGE), with 3 μL of each sample loaded into each well. Separation was carried out at 20 V/cm for 3 h in 0.5 $\times$ TBE running buffer. The gel was stained with 1 $\times$ SYBR Gold for 25 min and imaged using a ChemiDoc MP imaging system (Bio-Rad). The percent of digestion was calculated based on band intensity relative to the control sample.

CBSA-Based, EATR-Amplified Fluorescence Assay for Detection of DIS

Amounts of 2 μL of 100 μM DIS-LF or DIS-LF-536, 1 μL of 100 μM FQ-DIS-SF or FQ-DIS-SF-536, and 92 μL of DIS binding buffer (10 mM Tris-HCl, 0.5 mM MgCl_2 , pH 7.4) or 50% urine diluted with the binding buffer were mixed with 5 μL of solution containing various concentrations of DIS in the wells of a 96-well plate. The fluorescence of each sample was first measured ($\lambda_{\text{ex/em}} = 648/668$ nm) after a 30 min incubation at room temperature. Then, 5 μL of 0.2 U/ μL Exo III was added into each well to initiate EATR. After a 30 min digestion at room temperature, the fluorescence of each sample was measured again. The signal gain was calculated by $(F - F_0)/F_0 \times 100\%$, where F_0 is the fluorescence of the split aptamer mixtures without DIS and F is the fluorescence of split aptamer mixtures with different concentrations of DIS. Error bars were calculated from standard deviation of signal gains from three individual measurements at each DIS concentration.

Synthesis of Gold Nanoparticles (AuNPs) Modified with Cocaine-Binding CBSA Short Fragment

The modification of the thiolated short fragment onto 13 nm AuNPs was performed using a previously published protocol,³⁰ at a 300:1 molar ratio of thiolated short fragment/AuNP. The modified AuNPs were then treated with DTT to regulate surface coverage. Specifically, 50 μL of 5 nM modified AuNPs were mixed with 20 μL of solution containing different concentrations of DTT in 10 mM Tris-HCl (pH 7.4). After a 30 min incubation, the solution was centrifuged at 28 000 rcf for 10 min. The supernatant was discarded to remove the excess DTT and displaced DNA strands, and the precipitated AuNPs were resuspended in 10 mM Tris-HCl (pH 7.4). This step was repeated twice. Finally, the modified AuNPs were resuspended in 5 μL of the same buffer to obtain a particle concentration of 50 nM.

Quantitation of the Surface Coverage of CBSA Short Fragments on AuNPs via DTT Displacement

We measured surface coverage of the modified AuNPs using a standard DTT displacement assay.³¹ The AuNPs were diluted to a final volume of 55 μL in Tris buffer (10 mM Tris-HCl, pH 7.4). An amount of 50 μL of AuNP solution was loaded into a well of a 384-well plate, and the absorbance was measured using a TECAN M1000 Pro. The concentration of AuNPs was calculated using Beer's law ($\epsilon = 2.7 \times 10^8 \text{ M}^{-1} \cdot \text{cm}^{-1}$). An amount of 50 μL of the modified AuNP solution was collected and mixed with 50 μL of 1.0 M DTT solution. After an overnight incubation at room temperature, the solution was centrifuged at 25 000 rcf for 10 min to remove the AuNP precipitate. An amount of 20 μL of the supernatant was then mixed with 80 μL of 0.625 $\times$ OliGreen solution to obtain a 0.5 $\times$ Oligreen concentration. An amount of 100 μL of supernatant-Oligreen mixture was loaded into a well of a 384-well microplate, and fluorescence was measured using a TECAN M1000 Pro ($\lambda_{\text{ex/em}} = 500/525 \text{ nm}$). DTT-displaced oligonucleotides were quantified using an established calibration curve derived from CBSA short fragments that had been subjected to the same DTT treatment.

CBSA-Based, Colorimetric EATR Assay for Detection of Cocaine

Amounts of 2.5 μL of short-fragment-modified AuNPs (50 nM), 1 μL of 2.5 μM long fragment (COC-LF), 2 μL of a solution with different cocaine concentrations or different cutting agents with final concentration of 50 μM , and 15.5 μL of reaction buffer (final concentrations: 10 mM Tris-HCl, 100 mM NaCl, 0.75 mM MgCl_2 , 0.1 mg/mL BSA, pH 7.4) were mixed in wells of a 384-well plate. After 30 min, 4 μL of 1.25 U/ μL Exo III was added into each well. Absorbance spectra from 400–800 nm were recorded every 5 min, with pictures taken by Nikon D800 after 20 min of Exo III digestion.

RESULTS AND DISCUSSION

Engineering of DIS-Binding CBSA

Yang et al. recently isolated aptamers for various steroids, including DIS, using a library-immobilized SELEX approach.³² DIS is one of the most abundant steroid hormones in urine. The concentration of DIS in healthy adults typically ranges from 1–20 μM ^{33,34} but can climb to the submillimolar range in patients with adrenal tumors.³⁵ The isolated DIS-binding aptamer (DISS.1-AT) demonstrated a K_D of 0.44 μM (Supporting Information, Figure S1), and we used DISS.1-AT as a starting point for our CBSA engineering strategy that we have described previously.²² First, we generated parent split aptamers by removing the poly(T)₃ loop region from DISS.1-AT and reducing the number of base pairs in stem 1 (Figure 1A). We then fabricated a DIS-binding CBSA by fusing two sets of these parent split aptamers as shown in Figure 1B. The engineered CBSA consists of a short fragment and a long fragment. To facilitate EATR, we replaced the adenosine at position 9 from the 5' end of the short fragment with a C3 spacer abasic site (labeled as X in Figure 1C). In the presence of DIS, these two fragments assemble to form DIS-CBSA-4536 complex (Figure 1D), where the C3 spacer abasic site is opposite a thymine within the long fragment. Exo III can cleave the short fragment at this duplexed abasic site using its apurinic endonuclease activity,²⁹ releasing the intact long fragment and DIS molecules.

Design of a CBSA-Based EATR-Amplified Fluorescence Assay

To confirm binding cooperativity of DIS–CBSA-4536, we modified the short fragment with a Cy5 fluorophore and an Iowa Black RQ quencher at the 3′ and 5′ terminus, respectively (FQ–DIS-SF) (Figure 1D). In the absence of the target, both FQ–DIS-SF and the corresponding long fragment (DIS-LF) remain separate, and the fluorophore is quenched due to its close proximity with the quencher (Figure 2A). Upon the addition of DIS, the two fragments assemble to form a rigid CBSA–target complex, separating the fluorophore from the quencher and resulting in elevated fluorescence (Figure 2B). By titrating various DIS concentrations, we determined a Hill coefficient (n_H)³⁶ of 1.6 for DIS–CBSA-4536 (Supporting Information, Figure S2A), indicating considerable positive cooperativity. This implies that a transition of CBSA assembly from 10% to 90% requires only a 16-fold increase in target concentration, compared to its parent split aptamers which require an 81-fold increase.²⁸ NUPACK analysis³⁷ of the DIS-binding CBSA sequences demonstrated that both fragments are predominantly un-assembled (99%) in the absence of target under our experimental conditions, but DIS-SF exists primarily as a duplexed structure (Supporting Information, Figure S3A). Therefore, binding of the first target to DIS–CBSA-4536 should open up the DIS-SF duplex, facilitating binding of a second DIS molecule to the CBSA. This results in a high level of cooperativity. A $K_{1/2}$ ³⁶ of 491 μM was obtained, where $K_{1/2}$ represents the DIS concentration at which half of the binding domains are occupied (Supporting Information, Figure S2A). To demonstrate the highly sensitive target-induced assembly of the CBSA, we designed two sets of DIS-binding split aptamers with a single binding domain. Specifically, we extended stem 1 of the DIS-binding parent split aptamer (termed DIS-SA-36) (Figure 1B, right, and Supporting Information, Figure S2B) by one abasic site and five base pairs to form a split aptamer with a longer stem (termed DIS-SA-536) (Supporting Information, Figure S2C). The long fragments were unmodified while the short fragments were modified with a Cy5 fluorophore and an Iowa Black RQ quencher at the 3′ and 5′ terminus, respectively. We performed binding experiments with these two split aptamers along with the CBSA. DIS–CBSA-4536 demonstrated no background assembly in the absence of the target and progressive target-induced assembly with increasing concentrations of DIS. DIS-SA-536 also showed target-induced assembly with a moderately higher target affinity, but had high background assembly in the absence of DIS (Supporting Information, Figure S2A). As expected, no cooperativity was observed ($n_H = 1.05$) with this single-binding pocket split aptamer. The parent split aptamer, DIS-SA-36, was unable to assemble regardless of the presence or absence of target due to its thermal instability.

The affinity of DIS–CBSA-4536 was too low for direct detection of DIS at physiological levels.^{33,34} Nevertheless, the highly target-responsive feature of the CBSA enables us to demonstrate its compatibility with EATR-mediated signal amplification. We selected Exo III for this reaction because of its potent apurinic endonuclease activity, along with the fact that this enzyme is inexpensive and highly active at room temperature in a sequence-insensitive manner.^{38,39} When the CBSA–DIS complex assembles in the presence of DIS, Exo III is able to recognize the duplexed structure and specifically cleaves it at the abasic site (Figure 2C). This cleavage results in disassembly of the CBSA–target complex, releasing the long fragment, DIS molecules, and the fluorophore, which now fluoresces due to its separation

from the quencher. The released long fragment and target are then free to assemble with another FQ–DIS-SF (Figure 2D), starting the cycle anew. The end result is that a very small amount of target can be recycled for multiple rounds of assembly and cleavage, generating a substantially amplified fluorescence signal (Figure 2E).

Exo III Efficiently Mediates EATR of DIS–CBSA-4536 in Solution

We then set out to demonstrate efficient Exo III digestion of abasic-site-modified DIS-SF. In order to prevent nonspecific digestion³⁸ of the CBSA, we added a poly(A)₅ overhang at the 3' terminus of both fragments. We then performed a time-course of digestion with Exo III using DIS–CBSA-4536 in the presence or absence of DIS. The digestion products were collected after 5, 10, 15, 30, 60, and 120 min and analyzed by PAGE (Figure 3A). The concentration of retained short fragments was calculated relative to a control sample that was untreated with Exo III. We observed that Exo III cleavage of DIS-SF was rapid and specific, with 59% cleavage in the presence of 500 μ M DIS after 30 min. In contrast, only 9% of DIS-SF was cleaved in the absence of the target (Figure 3B). After 120 min, DIS-SF was almost completely cleaved (95%) in the DIS sample, compared to only 15% cleavage in the absence of the target (Figure 3B). These results confirmed that the apurinic endonuclease activity of Exo III could specifically cleave the CBSA even when it is bound to the DIS target. Notably, we observed moderate nonspecific digestion of DIS-LF by Exo III, resulting in partial removal of its poly(A)₅ protection (Figure 3A).³⁸ The total concentration of DIS-LF and its products, calculated from the intensity of all bands larger than 39 nt, remained constant after a 120 min digestion (Figure 3C). Since all digested products contained the intact recognition segments of the CBSA, we believe that EATR efficiency was not affected. We then demonstrated recycling of target and long fragment by performing an Exo III cleavage reaction with 1 μ M DIS-LF and 4 μ M DIS-SF in the presence of DIS. PAGE analysis confirmed that Exo III achieved efficient and specific cleavage of the short fragment, with 99% of DIS-SF cleaved after 240 min (Supporting Information, Figure S4A). In contrast, only 11% of DIS-SF was cleaved in the absence of DIS (Supporting Information, Figure S4B). We observed that less than 15% of DIS-LF was digested regardless of the presence or absence of target (Supporting Information, Figure S4C). On the basis of the fact that the short fragment was almost completely cleaved despite being present at a concentration greatly exceeding that of the long fragment, it is clear that Exo III-mediated recycling of the long fragment and target was taking place.

Sensitive DIS Detection in Biofluid Samples Using CBSA-Based EATR-Amplified Fluorescence Assay

Having demonstrated the feasibility of our CBSA-based EATR-amplified approach, we performed the fluorescence assay described in Figure 2 to detect DIS. As expected, when we incubated 1 μ M FQ–DIS-SF with 2 μ M DIS-LF and different concentrations of DIS for 30 min, we did not observe a significant DIS-induced fluorescence change even with 100 μ M DIS (Figure 4, parts A and B, 0 min), as target binding of the CBSA was too weak to efficiently assemble the CBSA–target complex. However, after adding 0.01 U/ μ L of Exo III, we saw a steady and concentration-dependent increase in fluorescence (Figure 4A). The initial rate at which fluorescence increased was proportional to the DIS concentration, and we found that concentrations as low as 1 μ M could be detected after 30 min of cleavage

(Figure 4B, inset). It should be noted that the fluorescence of the samples without DIS also increased to some extent due to the nonspecific digestion of FQ-DIS-SF (Figure 4A, 0 μM). These results clearly demonstrated that the sensitivity of our CBSA binding assay was enhanced 100-fold by implementing EATR, producing a measurable limit of detection that is approximately 500-fold lower than the $K_{1/2}$ of the CBSA. As a control experiment, we also performed our EATR-amplified fluorescence assay using DIS-SA-536. Although DIS-SA-536 underwent target-induced assembly and was efficiently cleaved by Exo III, we observed no target-induced signal amplification after a 30 min digestion because of the high levels of DIS-independent background assembly (Supporting Information, Figure S5, parts A and C). In contrast, DIS-CBSA-4536 exhibited clear target-induced signal amplification (Supporting Information, Figure S5, parts B and C), with rapid assembly occurring at very low concentrations of target and minimal background assembly in the absence of target. Importantly, our assay performed equally well in urine samples. Different concentrations of DIS were spiked into 50% diluted urine and tested with our CBSA fluorescence assay. After a 30 min enzyme digestion, we obtained a calibration curve with a linear range from 0 to 100 μM (Supporting Information, Figure S6A), which covers the urinary DIS concentration range in normal adults (1–50 μM). In addition, we found that concentrations as low as 1 μM DIS spiked in 50% urine could be distinguished from the background signal (Supporting Information, Figure S6B). Our results clearly demonstrated the robustness and sensitivity of our CBSA-based EATR fluorescence assay even in complex samples.

Design of the CBSA-Based Colorimetric Assay Employing EATR-Mediated AuNP Aggregation

Due to the highly responsive target-induced assembly feature of CBSAs, we believe that a similar EATR-amplified strategy can be readily adopted into different sensing platforms using other small-molecule-binding CBSAs. For example, the EATR-amplified assay described above is instrument-dependent, making it unsuitable for instrument-free applications. However, the same strategy can be also used to achieve rapid colorimetric detection of small-molecule targets in an instrument-free fashion. As a demonstration, we used the cocaine-binding CBSA previously developed by our group, COC-CBSA-5335, which has an n_H of 1.5 and a $K_{1/2}$ of 36 μM ,²² to develop an assay with a visual readout that is potentially suitable for field use. Specifically, we immobilized the short fragment of this CBSA (COC-SF) onto AuNPs, and then added the free long fragment (COC-LF) in solution (Figure 5A). In the presence of cocaine, the fragments assemble to form CBSA–cocaine complexes on the particle surface (Figure 5B). Exo III specifically recognizes the duplexed C3 spacer abasic site between the two binding domains and cleaves the AuNP-conjugated short fragment (Figure 5C), releasing the intact long fragment and cocaine for another round of target assembly and Exo III cleavage (Figure 5D). Once all of the short fragments have been cleaved from the particle surface (Figure 5E), the AuNPs lose their stability under the employed buffer conditions and rapidly aggregate, producing a visible red-to-blue color change (Figure 5F). The CBSA fragments cannot assemble in the absence of cocaine, precluding Exo III digestion, and the intact short fragments prevent AuNP aggregation such that the solution remains red.

EATR of CBSA Mediated by AP Endonuclease of Exo III Instead of Its 3′–5′ Exonuclease Activity

We first investigated the contribution of Exo III's 3′–5′ exonuclease activity to the digestion of CBSA–cocaine complexes relative to its endonucleolytic activity by engineering three derivatives of COC-SF. The first derivative (COC-SF-5′A) included a poly(A)₅ extension at the 5′ end that allows digestion of both the abasic site and the 3′ terminus (Figure 6A). For the second derivative, the poly(A)₅ protection was added at the 3′ instead of 5′ end (COC-SF-3′A), allowing cleavage only at the abasic site (Figure 6B). For the third derivative (COC-SF-T), we replaced the abasic site with a thymine, allowing digestion only from the 3′ terminus (Figure 6C). We also added a poly(A)₅ extension at the 5′ end of COC-SF-T to match the length of COC-SF-3′A. We performed Exo III digestion of a mixture of COC-LF with a 3′ poly(A)₅ modification combined with each of the three different short fragment derivatives in the presence or absence of 250 μM cocaine and characterized the digestion products using PAGE. For the COC-SF-5′A, we observed that 61% and 5% of the short fragment was cleaved with and without 250 μM cocaine, respectively, after a 5 min enzyme reaction (Supporting Information, Figure S7). After 60 min, the amount of cleaved COC-SF-5′A increased to 97% and 21%, respectively. We obtained a similar digestion profile for COC-SF-3′A, where Exo III cleavage occurs only at the abasic site. Amounts of 61% and 8% of the COC-SF-3′A were, respectively, cleaved with and without addition of cocaine after 5 min of digestion, which increased to 95% and 17%, respectively, after 60 min (Supporting Information, Figure S8). These results demonstrated that the apurinic endonuclease activity of Exo III is efficient enough to mediate the digestion of CBSA–cocaine complexes. Our control experiment with COC-SF-T demonstrated that Exo III was able to remove only one nucleotide from the short fragment. Only 5% of the COC-SF-T was digested both with and without cocaine after a 5 min Exo III reaction, and only 15% of the COC-SF-T was digested in the presence of cocaine versus 20% without cocaine after 60 min (Supporting Information, Figure S9). It is clear that the CBSA–cocaine complex inhibits the 3′-to-5′ exonuclease activity of Exo III. Thus, we concluded that the abasic site is sufficient to enable robust EATR signal amplification and poly(A)₅ protection at the 3′ end of the short fragment is unnecessary.

We then demonstrated that we could perform EATR with our cocaine-binding CBSA in solution. We performed a time-course of Exo III digestion using poly(A)₅ protected COC-LF and unprotected COC-SF, with a 1:8 ratio of COC-LF/COC-SF, either with or without 250 μM cocaine. PAGE analysis of the digestion products showed rapid and specific Exo III digestion, with more than 60% of the short fragment cleaved in the presence of cocaine after only 10 min (Figure 7). In contrast, less than 5% of the short fragment was cleaved in the absence of cocaine. After a 30 min reaction, COC-SF was almost completely digested (92%) in the presence of cocaine, while only 14% was digested in the absence of cocaine, demonstrating the specificity of EATR amplification in solution.

DNA Surface Coverage and the Length of COC-SF Affect EATR-Mediated AuNP Aggregation

We employed our CBSA-based EATR-amplified assay to achieve naked-eye detection of cocaine. We conjugated a thiolated version of COC-SF (Supporting Information, Table S1,

SH-COC-SF-1X) onto AuNPs as described previously.³⁰ Since DNA surface coverage has a critical influence on EATR-mediated AuNP aggregation, we developed a simple strategy for generating AuNPs displaying different quantities of short fragment. We did this by incubating short fragment-saturated AuNPs with various micromolar concentrations of DTT to displace different quantities of immobilized short fragment from the particle surface. We then measured the surface coverage resulting from treatment with various DTT concentrations by mixing each of these batches of AuNPs with an equal volume of 1.0 M DTT⁴¹ and incubating overnight to remove all remaining DNA strands from the particle surface. We subsequently quantified these by centrifuging the samples and measured the DNA concentration in the collected supernatant with OliGreen, a DNA binding dye.³¹ A calibration curve (Supporting Information, Figure S10A) determined that treatment with DTT concentrations of 0, 100, 200, 300, and 400 μM produced AuNPs that, respectively, displayed 102 ± 1 , 41 ± 1 , 34 ± 1 , 25 ± 1 , and 22 ± 1 strands/particle (Supporting Information, Figure S10B). This demonstrates that DTT treatment can effectively regulate DNA surface coverage on AuNPs in a concentration-dependent manner. AuNPs treated with 500 μM DTT yielded a surface coverage of 20 ± 1 strands/particle; the resulting particles were unstable and spontaneously aggregated in the reaction buffer (data not shown). Thus, these AuNPs were omitted from following experiments.

We then assessed how differing levels of surface coverage affect CBSA-cocaine assembly, exonuclease cleavage, and AuNP aggregation. Since the CBSA will be assembled on the particle, Exo III will have minimal accessibility to the 3' end of the long fragment. We incubated our various batches of 5 nM SH-COC-SF-1X-modified AuNPs with 100 nM unprotected COC-LF in the presence or absence of 250 μM cocaine for 30 min, and then added 0.2 U/ μL Exo III. We tracked the signal response by performing time-dependent measurements of UV-vis absorbance. Well-dispersed AuNPs have an absorbance peak at 520 nm; as AuNPs start to aggregate, this peak decreases and a new peak appears at 650 nm.⁴² Therefore, we used the ratio of A_{650}/A_{520} to evaluate EATR-mediated AuNP aggregation. When the value of A_{650}/A_{520} is below 0.4, the AuNPs are well-separated and the solution appears red. When the value of A_{650}/A_{520} is 0.6, the AuNPs begin to aggregate and the solution appears purple. A value of $A_{650}/A_{520} = 0.8$ indicates complete aggregation, and the solution appears dark blue. We found that SH-COC-SF-1X-modified AuNPs with relatively low surface coverage (25 strands/particle) demonstrated much faster aggregation than AuNPs with high surface coverage (102 strands/particle) (Supporting Information, Figure S11). We observed no aggregation for these 102 strands/particle AuNPs, even 70 min after the addition of Exo III (Supporting Information, Figure S11A). Likewise, we did not observe a visible color change at a surface density of 41 strands/particle (Supporting Information, Figure S11B). However, further reductions in surface coverage produced a clear increase in the rate of AuNP aggregation, and AuNPs with surface coverages of 34, 25, and 22 strands/particle produced a dark blue color ($A_{650}/A_{520} = 0.8$) within 72, 52, and 38 min of Exo III digestion, respectively. This indicates both specific CBSA-cocaine assembly and successful EATR amplification. In the meantime, the AuNPs remained well-dispersed at all levels of surface coverage and all target-free samples remained red ($A_{650}/A_{520} = 0.4$) (Supporting Information, Figure S11C-E). Notably, both target- and nontarget-mediated Exo III digestion and AuNP aggregation are more pronounced for AuNPs with lower surface

coverages. We identified 25 strands/particle as the optimal level of surface coverage for our assay due to the existence of detection window during which the drug-containing sample is blue while the drug-free sample remains red (Supporting Information, Figure S11F). Although the ratio between COC-LF and AuNP-conjugated SH-COC-SF-1X under this optimal surface coverage was close to 1:1, we believe that Exo III-mediated recycling of LF and target was taking place on the particle surface since DNA hybridization efficiency on AuNPs is usually below 20%.^{43,44} We believe that our DTT-based strategy for regulating DNA surface coverage plays multiple roles in facilitating exonuclease digestion of AuNP-conjugated short fragments for rapid cocaine detection. First, it prevents nonspecific binding of DNA bases⁴⁵ by occupying vacant areas on the particle surface with DTT, lifting the covalently bound aptamer fragments into an upright orientation that increases CBSA-target assembly and enzyme accessibility.⁴⁶ Second, it adjusts the surface density by replacing some of the chemically bound aptamer fragments. This significantly reduces the steric and electrostatic repulsion caused by neighboring DNA strands, enabling optimized CBSA-cocaine complex assembly.⁴⁷ Finally, by decreasing DNA surface coverage, this treatment also decreases local salt concentration at the surface,⁴⁸ which greatly reduces the salt-induced inhibition of Exo III.³⁹

We predicted that a longer version of COC-SF should further increase the stability of our modified AuNPs by suppressing nonspecific digestion-mediated aggregation. Thus, we designed a new construct (Supporting Information, Table S1, SH-COC-SF-2X) comprising two tandem abasic-site-incorporated short fragment repeats with a (T)₆ linker. Our reasoning was that the assay's specificity should be enhanced by this change, since Exo III will have to cleave both abasic sites in order to trigger cocaine recycling and AuNP aggregation (Supporting Information, Figure S12). We modified 13 nm AuNPs with SH-COC-SF-2X, used our DTT regulation strategy to adjust the surface coverage of AuNPs, and tested the effects of DNA surface coverage on EATR performance. A calibration curve (Supporting Information, Figure S13A) determined that treatment with DTT concentrations of 0, 200, 300, 400, and 500 μ M produced AuNPs that, respectively, displayed 83 ± 2 , 23 ± 1 , 20 ± 1 , 18 ± 1 , and 16 ± 1 strands per particle (Supporting Information, Figure S13B). As expected, increasing concentrations of DTT produced AuNPs with lower levels of surface coverage (Supporting Information, Figure S13). Compared to SH-COC-SF-1X-modified AuNPs, SH-COC-SF-2X-modified AuNPs are stable even with surface coverages as low as 16 strands/particle. This is most likely due to the increased number of negatively charged DNA phosphate groups located on the AuNP surface, providing more repulsion among particles. Lower-coverage AuNPs underwent EATR-mediated AuNP aggregation at a much faster rate than those with saturating coverage (Supporting Information, Figure S14A–E). The surface coverage of 20 ± 1 strands/particle was selected for a combination of short reaction time and long detection window (Supporting Information, Figure S14F).

Visual Detection of Cocaine Using CBSA-Based EATR-Amplified Colorimetric Assay

Finally, we compared the assay's performance with SH-COC-SF-1X versus SH-COC-SF-2X at optimized surface coverage. SH-COC-SF-2X-modified AuNPs aggregated more rapidly than those modified with SH-COC-SF-1X (Supporting Information, Figure S15). We then used SH-COC-SF-2X-modified AuNPs to test the sensitivity of our assay. The

particles remained stable in the reaction buffer, and the color of the sample was not affected by cocaine concentrations of up to 500 μM in the absence of Exo III (Figure 8A, 0 min). Upon addition of Exo III, samples containing cocaine demonstrated different levels of particle aggregation, resulting in a distinctive color readout that shifted from light purple, dark purple, to dark blue at increasing drug concentrations over the course of 20 min (Figure 8A, 20 min), with A_{650}/A_{520} varying from 0.39 (0.2–1 μM) to 0.90 (500 μM). The color of the cocaine-free sample remained unchanged. We could observe a clear blue color in samples containing as little as 10 μM cocaine with the naked eye after 20 min (Figure 8A). Using UV–vis spectroscopy measurements, we obtained a measurable limit of detection of 2 μM (Figure 8B). We determined the target specificity of the CBSA-based EATR-amplified colorimetric assay by challenging it with cocaine along with common cutting agents observed in street samples⁴⁹ such as lidocaine, caffeine, levamisole, and benzocaine at a concentration of 50 μM . We observed a red-to-blue color change only in the cocaine sample within 20 min (Supporting Information, Figure S16). These results show that our EATR colorimetric assay can be used for the specific and sensitive naked-eye detection of cocaine in seized samples.

CONCLUSIONS

We have demonstrated a general and simple approach for developing a variety of CBSA-based EATR-amplified assays for the sensitive detection of small-molecule targets. The 3′–5′ exonuclease activity of Exo III has been used extensively in DNA-based EATR assays,⁴ but our results demonstrated that Exo III's 3′–5′ exonuclease activity is greatly inhibited by the tertiary structure of target–CBSA complexes. To achieve rapid CBSA-based EATR, we incorporated a C3 spacer abasic site between the two target-binding domains on our CBSA short fragment, creating a recognition site for Exo III's apurinic endonuclease activity. We have previously demonstrated that the apurinic endonuclease activity of Exo III can effectively enable EATR-mediated AuNP aggregation for rapid and sensitive detection of single-base-mismatched DNA.⁶ Here, we showed that Exo III's apurinic endonuclease activity could mediate CBSA-based EATR in solution as well as on particle surface. We thus believe that the highly active and robust apurinic endonuclease activity of Exo III should allow many DNA-based EATR sensor platforms to be applied to our CBSA-based EATR assays for detection of various small-molecule targets.

As an initial demonstration, we engineered a DIS-binding CBSA with a duplexed C3 spacer abasic site incorporated between the two target-binding domains. This allowed us to exploit EATR signal amplification, which enhanced the sensitivity of our CBSA-based fluorescence assay by 100-fold relative to an unamplified assay. The detection limit of our CBSA-based EATR-amplified fluorescence assay is 100 and 50 times lower than previously reported DIS assays based on a split aptamer²¹ and a structure-switching aptamer,⁵⁰ respectively. This assay also allowed us to detect DIS at concentrations as low as 1 μM in 50% urine within 30 min. We then demonstrated that this strategy could be adopted for rapid colorimetric detection of cocaine, based on EATR-mediated aggregation of AuNPs—a useful strategy for instrument-free detection. This assay enabled us to achieve rapid, naked-eye detection of cocaine at low-micromolar concentrations within 20 min and is more sensitive, robust, or

simple compared to previously reported methods for the visual detection of cocaine (Supporting Information, Table S2).

Recent work has described SELEX approaches for isolating small-molecule-binding aptamers with a three-way junction structure using a partially structured library.³² These aptamers can be readily engineered into CBSAs using the strategy demonstrated in this work, and as also shown here, these CBSAs can then be readily adapted for use in EATR-amplified assays. On the basis of the generality of our CBSA engineering procedure and excellent performance of EATR assays in complex samples, we believe that the strategy described here will accelerate the development of sensitive and accurate sensors for detecting small-molecule targets in fields including environmental monitoring, food safety, law enforcement, medical diagnostics, and public health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health—National Institute on Drug Abuse [R15DA036821] and National Institute of Justice, Office of Justice Programs, U.S. Department of Justice Awards (2013-DN-BX-K032 and 2015-R2-CX-0034). The opinions, findings, and conclusions or recommendations expressed in this publication/program/exhibition are those of the author(s) and do not necessarily reflect those of the Department of Justice.

References

1. Rogers KR. *Mol Biotechnol.* 2000; 14:109–129. [PubMed: 10872504]
2. Duan RX, Lou XD, Xia F. *Chem Soc Rev.* 2016; 45:1738–1749. [PubMed: 26812957]
3. Li JJ, Chu YZ, Lee BYH, Xie XS. *Nucleic Acids Res.* 2008; 36:e36. [PubMed: 18304948]
4. Gerasimova YV, Kolpashchikov DM. *Chem Soc Rev.* 2014; 43:6405–6438. [PubMed: 24901032]
5. Zuo XL, Xia F, Xiao Y, Plaxco KW. *J Am Chem Soc.* 2010; 132:1816–1818. [PubMed: 20092337]
6. Wu S, Liang PP, Yu HX, Xu XW, Liu Y, Lou XH, Xiao Y. *Anal Chem.* 2014; 86:3461–3467. [PubMed: 24611947]
7. Gerasimova YV, Peck S, Kolpashchikov DM. *Chem Commun.* 2010; 46:8761–8763.
8. Wu D, Yin BC, Ye BC. *Biosens Bioelectron.* 2011; 28:232–238. [PubMed: 21820885]
9. Lyamichev V, Mast AL, Hall JG, Prudent JR, Kaiser MW, Takova T, Kwiatkowski RW, Sander TJ, de Arruda M, Arco DA, Neri BP, Brow MAD. *Nat Biotechnol.* 1999; 17:292–296. [PubMed: 10096299]
10. Tuerk C, Gold L. *Science.* 1990; 249:505–510. [PubMed: 2200121]
11. Lee JF, Hesselberth JR, Meyers LA, Ellington AD. *Nucleic Acids Res.* 2004; 32:D95–100. [PubMed: 14681367]
12. Mairal T, Özalp VC, Lozano Sánchez P, Mir M, Katakis I, O'Sullivan CK. *Anal Bioanal Chem.* 2008; 390:989–1007. [PubMed: 17581746]
13. Song KM, Lee S, Ban C. *Sensors.* 2012; 12:612–631. [PubMed: 22368488]
14. Lee JH, Yigit MV, Mazumdar D, Lu Y. *Adv Drug Delivery Rev.* 2010; 62:592–605.
15. Nováková L, Vl ková H. *Anal Chim Acta.* 2009; 656:8–35. [PubMed: 19932811]
16. Toh SY, Citartan M, Gopinath SCB, Tang TH. *Biosens Bioelectron.* 2015; 64:392–403. [PubMed: 25278480]
17. Citartan M, Gopinath SCB, Tominaga J, Tan SC, Tang TH. *Biosens Bioelectron.* 2012; 34:1–11. [PubMed: 22326894]

18. Stojanovic MN, de Prada P, Landry DW. *J Am Chem Soc.* 2000; 122:11547–11548. [PubMed: 29048887]
19. Zou RX, Lou XH, Ou HC, Zhang Y, Wang WJ, Yuan M, Guan M, Luo ZF, Liu YY. *RSC Adv.* 2012; 2:4636–4638.
20. Chen AL, Yan MM, Yang SM. *TrAC, Trends Anal Chem.* 2016; 80:581–593.
21. Kent AD, Spiropoulos NG, Heemstra JM. *Anal Chem.* 2013; 85:9916–9923. [PubMed: 24033257]
22. Yu HX, Canoura J, Guntupalli B, Lou XH, Xiao Y. *Chem Sci.* 2017; 8:131–141. [PubMed: 28451157]
23. Zheng DM, Zou RX, Lou XH. *Anal Chem.* 2012; 84:3554–3560. [PubMed: 22424113]
24. Biebricher AS, Heller I, Roijmans RFH, Hoekstra TP, Peterman EJG, Wuite GJL. *Nat Commun.* 2015; 6:7304. [PubMed: 26084388]
25. Kang S, Ohshima K, Shimizu M, Amirhaeri S, Wells RD. *J Biol Chem.* 1995; 270:27014–27021. [PubMed: 7592950]
26. Tian LG, Sayer JM, Kroth H, Kalena G, Jerina DM, Shuman S. *J Biol Chem.* 2003; 278:9905–9911. [PubMed: 12524450]
27. Pierce JR, Nazimiec M, Tang MS. *Biochemistry.* 1993; 32:7069–7078. [PubMed: 8343501]
28. Simon AJ, Vallée-Bélisle A, Ricci F, Plaxco KW. *Proc Natl Acad Sci U S A.* 2014; 111:15048–15053. [PubMed: 25288724]
29. Takeuchi M, Lillis R, Demple B, Takeshita M. *J Biol Chem.* 1994; 269:21907–21914. [PubMed: 7520446]
30. Yu HX, Xu XW, Liang PP, Loh KY, Guntupalli B, Roncancio D, Xiao Y. *Bioconjugate Chem.* 2017; 28:933–943.
31. Liu CW, Huang CC, Chang HT. *Langmuir.* 2008; 24:8346–8350. [PubMed: 18582003]
32. Yang KA, Pei R, Stefanovic D, Stojanovic MN. *J Am Chem Soc.* 2012; 134:1642–1647. [PubMed: 22142383]
33. Straub RH, Weidler C, Demmel B, Herrmann M, Kees F, Schmidt M, Schölmerich J, Schedel J. *Ann Rheum Dis.* 2004; 63:961–968. [PubMed: 15249323]
34. Strahm E, Kohler I, Rudaz S, Martel S, Carrupt PA, Veuthey JL, Saugy M, Saudan C. *J Chromatogr A.* 2008; 1196–1197:153–160.
35. Mason HL, Kepler EJ. *J Biol Chem.* 1945; 161:235–257. [PubMed: 21005731]
36. Hill AV. *J Physiol.* 1910; 40:190–224. [PubMed: 16993004]
37. Zadeh JN, Steenberg CD, Bois JS, Wolfe BR, Pierce MB, Khan AR, Dirks RM, Pierce NA. *J Comput Chem.* 2011; 32:170–173. [PubMed: 20645303]
38. Shida T, Noda M, Sekiguchi J. *Nucleic Acids Res.* 1996; 24:4572–4576. [PubMed: 8948651]
39. Hoheisel JD. *Anal Biochem.* 1993; 209:238–246. [PubMed: 8470795]
40. Scientific Working Group for Forensic Toxicology. *J Anal Toxicol.* 2013; 37:452–474. [PubMed: 23934984]
41. Hurst SJ, Lytton-Jean AKR, Mirkin CA. *Anal Chem.* 2006; 78:8313–8318. [PubMed: 17165821]
42. Link S, El-Sayed MA. *J Phys Chem B.* 1999; 103:4212–4217.
43. Demers LM, Mirkin CA, Mucic RC, Reynolds RA, Letsinger RL, Elghanian R, Viswanadham G. *Anal Chem.* 2000; 72:5535–5541. [PubMed: 11101228]
44. Nicewarner Peña SR, Raina S, Goodrich GP, Fedoroff NV, Keating CD. *J Am Chem Soc.* 2002; 124:7314–7323. [PubMed: 12071740]
45. Parak WJ, Pellegrino T, Micheel CM, Gerion D, Williams SC, Alivisatos AP. *Nano Lett.* 2003; 3:33–36.
46. Wu J, Campuzano S, Halford C, Haake DA, Wang J. *Anal Chem.* 2010; 82:8830–8837. [PubMed: 20883023]
47. Cederquist KB, Keating CD. *Langmuir.* 2010; 26:18273–18280. [PubMed: 21038880]
48. Seferos DS, Prigodich AE, Giljohann DA, Patel PC, Mirkin CA. *Nano Lett.* 2009; 9:308–311. [PubMed: 19099465]
49. de Souza LM, Rodrigues RRT, Santos H, Costa HB, Merlo BB, Filgueiras PR, Poppi RJ, Vaz BG, Romão W. *Sci Justice.* 2016; 56:73–79. [PubMed: 26976463]

50. Peterson AM, Jahnke FM, Heemstra JM. *Langmuir*. 2015; 31:11769–11773. [PubMed: 26465173]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Design of DIS-binding CBSA. (A) The single-stranded DIS binding aptamer (DISS.1-AT) was truncated at stem 1 and stem 2 to generate the parent split aptamers. (B) Two sets of parent split aptamers were (C) merged and modified to incorporate (D) a C3 spacer abasic site (denoted by X) that resides opposite a thymine nucleotide when the CBSA duplex is assembled. A fluorophore (Cy5) and a quencher (Iowa Black RQ) were, respectively, modified at the 3' and 5' terminus of the short CBSA fragment. A poly(A)₅ overhang was added on the 3' termini of each CBSA fragment to prevent nonspecific Exo III digestion.

Figure 2.

Working principle of our CBSA-based, EATR-amplified fluorescence assay. (A) FQ-DIS-SF contains a fluorophore-quencher pair and remains dark in the absence of target. (B) In the presence of DIS, the CBSA assembles (C), creating a duplex that can be recognized and cleaved by Exo III at the abasic site (denoted by X). (D) The complex then disassembles, releasing the intact long fragment and target for additional rounds of complex formation and digestion. The fluorophore is also released and produces a signal, which (E) becomes amplified over the course of many rounds of EATR.

Figure 3.

Time-course of Exo III-mediated cleavage of DIS-CBSA-4536. (A) PAGE analysis of digestion products from DIS-CBSA-4536. Reactions consisted of 1 μ M DIS-SF, 1 μ M DIS-LF, and 0.004 U/ μ L Exo III with or without 500 μ M DIS after 5, 10, 15, 30, 60, and 120 min of digestion. Cleavage of short (B) and long (C) fragments was quantified by $(Int_0 - Int)/Int_0 \times 100\%$, where Int is the band intensity after the addition of Exo III and Int_0 is the band intensity before the addition of Exo III.

Figure 4.

CBSA-based EATR-amplified fluorescence assay for sensitive detection of DIS. (A) Fluorescence time-course measurements after adding 0.01 U/ μ L of Exo III to our CBSA in the presence of different concentrations of DIS (0, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 μ M). (B) Calibration curves before (black) and 30 min after (red) EATR signal amplification. The inset shows the calibration curve at low DIS concentrations. The signal gain was calculated from the fluorescence intensity of the samples. Error bars represent the standard deviation of three measurements. Limit of detection is determined using the lowest nonzero calibrator concentration (ref 40).

Figure 5.

Colorimetric CBSA-based EATR-amplified assay for naked-eye detection of cocaine. (A) AuNPs are coupled to the short fragment (COC-SF), creating steric repulsion between AuNPs that produces a visible red color. Upon addition of long fragment (COC-LF) and cocaine (B), the CBSA assembles, and the resulting duplexed abasic site can then (C) be digested by Exo III. (D) This cleaves off COC-SF while recycling COC-LF and cocaine for another round of assembly and digestion, until (E) all short fragments have been sheared from the AuNP. (F) These sheared AuNPs can now aggregate, producing a visible red-to-blue color change.

Figure 6. Cocaine-binding CBSA with a poly(A)₅-protected long fragment and different derivatives of the short fragment. Arrows indicate suitable digestion sites for Exo III.

Figure 7.

Time-course of Exo III digestion of cocaine-binding CBSA. (A) Digestion of a mixture of 1 μM COC-LF and 8 μM COC-SF by 0.1 U/ μL Exo III in the presence or absence of 250 μM cocaine after 5, 10, 15, 30, or 60 min of digestion. (B) Digestion was quantified as described above in Figure 3B.

Figure 8.

CBSA assay for cocaine detection with SH-COC-SF-2X-conjugated AuNPs. (A) After 20 min, samples containing cocaine at concentrations $10 \mu\text{M}$ produced a clearly visible red-to-blue color change. Cocaine-free samples without cocaine remained red. (B) Calibration curve of our colorimetric CBSA-based cocaine assay; inset shows the calibration curve at low cocaine concentrations.