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No Structure-Switching Required: A Generalizable Exonuclease-Mediated Aptamer-Based Assay for Small-Molecule Detection

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Abstract

The binding of small molecules to double-stranded DNA can modulate its susceptibility to digestion by exonucleases. Here, we show that the digestion of aptamers by exonuclease III can likewise be inhibited upon binding of small-molecule targets and exploit this finding for the first time to achieve sensitive, label-free small-molecule detection. This approach does not require any sequence engineering and employs prefolded aptamers which have higher target-binding affinities

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ASSOCIATED CONTENT

Supporting Information

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Characterization of target-binding affinity of DIS-binding aptamers using ITC; aptamer mutant DIS-37-M and its binding affinity to DIS; PAGE analysis of exonuclease-treated DIS-37 and DIS-37-M after a 15 min reaction in the absence or presence of DIS; quantification of the digestion product of DIS-37 generated by the exonuclease mixture with 0–250 μ M DIS via staining with SYBR Gold; quantification of the digestion product of DIS-37 generated by the exonuclease mixture with 0–10 μ L DIS via staining with SYBR Gold; schematic of dual-exonuclease-mediated COC-38 digestion in the absence and presence of cocaine; characterization of target-binding affinity of cocaine-binding aptamers and a mutant using ITC; target concentration-dependent inhibition of dual-exonuclease-mediated ATP-33 digestion in the absence and presence of ATP; characterization of target binding affinity of ATP-binding aptamers and a mutant using ITC; target concentration-dependent inhibition of dual-exonuclease-mediated digestion of an ATP-binding aptamer and its mutant after a 20 min reaction; design of molecular beacons for the ATP- and cocaine-binding aptamer digestion products produced by the exonuclease mixture; calibration curves of the dual-exonuclease-mediated molecular-beacon-based multiplex assay for the detection of cocaine and ATP; comparison of the performance of the molecular-beacon-based multiplex assay with individual exonucleases versus both; comparison of various aptamer-based assays for detection of cocaine or ATP (PDF)

The authors declare no competing financial interest.

than structure-switching aptamers widely used in current small-molecule detecting assays. We first use a dehydroisoandrosterone-3-sulfate-binding aptamer to show that target binding halts exonuclease III digestion four bases prior to the binding site. This leaves behind a double-stranded product that retains strong target affinity, whereas digestion of nontarget-bound aptamer produces a single-stranded product incapable of target binding. Exonuclease I efficiently eliminates these single-stranded products but is unable to digest the target-bound double-stranded product. The remaining products can be fluorescently quantified with SYBR Gold to determine target concentrations. We demonstrate that this dual-exonuclease-mediated approach can be broadly applied to other aptamers with differing secondary structures to achieve sensitive detection of various targets, even in biological matrices. Importantly, each aptamer digestion product has a unique sequence, enabling the creation of multiplex assays, and we successfully demonstrate simultaneous detection of cocaine and ATP in a single microliter volume sample in 25 min via sequence-specific molecular beacons. Due to the generality and simplicity of this assay, we believe that different DNA signal-reporting or amplification strategies can be adopted into our assay for target detection in diverse analytical contexts.

INTRODUCTION

Aptamers are nucleic acid-based affinity reagents, isolated via in vitro systematic evolution of ligands by exponential enrichment (SELEX).^{1,2} They have gained considerable attention as excellent biorecognition elements, with diverse applications in areas such as drug screening,³ environmental monitoring,⁴ molecular and cell imaging,^{5–7} and medical diagnostics.^{8,9} This is in part because aptamers can be isolated for essentially any target, including metal ions, small molecules, proteins, or whole cells.^{10,11} Aptamers have desirable properties such as high chemical stability and long shelf life and can be chemically synthesized in an inexpensive and reproducible manner.^{10,11} These advantages make aptamers ideal for use in biosensors. Aptamer-based assays have been developed for the detection of a multitude of targets, particularly small molecules.^{12,13} Most aptamer-based assays are based on structure-switching aptamers which undergo large conformational changes to report the presence of the target.^{3,14,15} Upon binding to the sensing aptamer, the target induces a specific folding event that produces a colorimetric,¹⁶ fluorescent,¹⁵ or electrochemical¹⁷ readout. However, most SELEX methods use unstructured libraries and therefore yield aptamers that have unpredictable binding domains and no structure-switching functionality.^{14,18} Thus, additional engineering steps are typically required to introduce structure-switching functionality into such aptamers.^{3,14,15} Specifically, truncation must first be performed to identify the target-binding domain of the aptamer and then to destabilize it so that the aptamer can undergo a large target-induced conformational change. To report this change, the truncated aptamer must be chemically modified with a signal-reporting element such as a fluorophore,³ electroactive tag,¹⁷ enzyme,¹⁹ or quantum dot.²⁰ This sensing strategy is labor intensive and costly, and destabilization of the aptamer greatly reduces its target-binding affinity.^{21,22} Strand displacement strategies offer an alternative for small-molecule detection, in which the target-binding domain of an aptamer is identified and then partially blocked via hybridization to a complementary DNA (cDNA) strand. Detection relies on target-induced displacement of the cDNA and quantification of either the displaced cDNA²³ or the target-aptamer complex.¹⁴ Since aptamers generally have higher affinity for

the cDNA strand than the small-molecule target, strand-displacement methods are hindered by competition between the cDNA and the target for binding to the aptamer, which reduces target-binding affinity and undermines sensor performance.^{24,25}

To overcome the limitations described above, we developed a generalizable aptamer-based assay based on target-induced exonuclease inhibition to detect small-molecule targets without the need for binding-induced conformational changes. Previous studies have shown that exonuclease III (Exo III), a 3'-to-5' double-strand DNA exonuclease,²⁶ is sensitive to local structural changes in double-stranded DNA induced by small-molecule binding and that such binding can halt digestion a few bases before the binding site.²⁷⁻²⁹ We recently found that this phenomenon also holds true for small-molecule binding aptamers and exploited this finding to introduce structure-switching functionality into fully folded aptamers.³⁰ Here, we develop an assay that utilizes high-affinity, prefolded aptamers and two exonucleases, Exo III and exonuclease I (Exo I), to detect small molecules. Favorably, this assay does not require any sequence engineering, truncation, or labeling. As proof of concept, we performed Exo III digestion of a dehydroisoandrosterone-3-sulfate (DIS)-binding 37-nt aptamer,³¹ DIS-37, which has a three-way-junction-structured binding domain and a duplexed, blunt-ended 3' terminus. Exo III catalyzed the 3'-to-5' digestion of the double-stranded stem of DIS-37, removing three nucleotides to form a 34-nt product regardless of the presence or absence of DIS. Further digestion of the target-bound 34-nt product was halted in the presence of DIS, but in the absence of DIS, the 34-nt product was further digested to produce a single-stranded 30-nt product that was resistant to Exo III digestion. We found that Exo I, a 3'-to-5' single-strand exonuclease,³² was able to degrade this 30-nt single-stranded, nontarget-bound digestion product into mononucleotides but was incapable of digesting the 34-nt double-stranded digestion product of the target-bound aptamer. We therefore used a mixture of Exo III and Exo I to develop a fluorescence assay to detect DIS using SYBR Gold, a DNA-binding dye,³³ as a signal reporter. Our assay proved to be very sensitive and had low background, achieving a detection limit of 500 nM DIS in 50% urine within 15 min. We further demonstrated the generality of this fluorescence assay with a three-way-junction-structured cocaine-binding aptamer and a hairpin-structured ATP-binding aptamer. We observed that minimal signal was generated in the absence of target due to the fact that both exonucleases completely digested the nontarget-bound aptamers. In contrast, a large signal was produced in the presence of target as exonuclease digestion of target-bound aptamers was strongly inhibited. Notably, our assay is highly robust as it maintains excellent performance in a variety of buffer conditions and biological sample matrices.

Finally, we demonstrated our assay's potential for single-pot, multiplex small-molecule detection by exploiting the fact that the digestion product of each aptamer-target pair has a unique sequence that can be specifically recognized by an antisense DNA probe. As a demonstration, we used fluorophore-quencher-modified molecular beacons to quantify the digestion products produced by the exonuclease mixture to simultaneously detect ATP and cocaine. Due to the high target specificity of the aptamers and high sequence specificity of the molecular beacons, we were able to simultaneously quantify both targets in microliter-volume samples without any cross talk in 25 min. We believe our assay should be broadly generalizable and can be combined with different DNA signal-reporting strategies for

multiplexed small-molecule detection in applications such as medical diagnostics, drug screening, food safety, environmental monitoring, and biodefense.

MATERIALS AND METHODS

Reagents.

Exonuclease III and exonuclease I (both from *E. coli*) were purchased from New England Biolabs. Adenosine-5'-triphosphate (ATP) disodium salt trihydrate was purchased from MP Biomedical. Formamide was purchased from Fisher Scientific. SYBR Gold was purchased from Invitrogen. Cocaine hydrochloride, dehydroisoandrosterone-3-sulfate, and all other chemicals were purchased from Sigma-Aldrich unless otherwise specified. All oligonucleotides were ordered from Integrated DNA Technologies and purified with HPLC. COC-MB was purchased from LGC Biosearch Technologies with HPLC purification. Oligonucleotides were dissolved in PCR-quality water, and the concentrations were measured using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). The following DNA sequences were employed in this work (mutated nucleotides are underlined):

DIS-37:5'-TCG GGA CGT GGA TTT TCC GCA TAC GAA GTT GTC CCG
A-3'

DIS-34:5'-TCG GGA CGT GGA TTT TCC GCA TAC GAA GTT GTC C-3'

DIS-33:5'-TCG GGA CGT GGA TTT TCC GCA TAC GAA GTT GTC-3'

DIS-30:5'-TCG GGA CGT GGA TTT TCC GCA TAC GAA GTT-3'

DIS-37-M: 5'-TCG GGA CGT GGA TTT TCC GCC TAC GAA GTG GTC CCG
A-3'

ATP-33:5'-CGC ACC TGG GGG AGT ATT GCG GAG GAA GGT GCG-3'

ATP-30:5'-CGC ACC TGG GGG AGT ATT GCG GAG GAA GGT-3'

ATP-25:5'-CGC ACC TGG GGG AGT ATT GCG GAG G-3'

ATP-33-M: 5'-CGC ACC TGG GGA AGT ATT GCG GTG GAA GGT GCG-3'

COC-38:5'-GGG TGA CAA GGA AAA TCC TTC AAT GAA GTG GGT CAC
CC-3'

COC-35:5'-GGG TGA CAA GGA AAA TCC TTC AAT GAA GTG GGT CA-3'

COC-33:5'-GGG TGA CAA GGA AAA TCC TTC AAT GAA GTG GGT-3'

COC-38-M: 5'-GGG TGA CAA GGA AAA TCC TTC GAT GAA GTG GGT
CAC CC-3'

COC-MB: 5'-/56-FAM/GCG AGC ACC CAC TTC ATT GAA GGA TTT TCC
TTG TCA CCC GCT CGC/BHQ-1/-3'

ATP-MB: 5'-/5Cy5/GCG AGC CTT CCT CCG CAA TAC TCC CCC AGG TGC
GGC TCG C/3IAbRQSp/-3'

(a, /56-FAM/represents FAM; b, /5Cy5/represents Cy5; c, /BHQ-1/represents Blackhole Quencher 1; d, /3IAbRQSp/represents Iowa Black RQ)

Aptamer Digestion Experiments.

Aptamer digestion experiments were performed using the following procedure unless otherwise specified. A 1 μL amount of 50 μL aptamer or mutant aptamer was added into 44 μL of reaction buffer containing various concentrations of the appropriate target in a 200 μL PCR tube. The buffer composition varied for each aptamer–target pair, with final concentrations for each reaction as follows: DIS (20 mM Tris, 25 mM MgCl_2 , 1% DMSO, 0.1 mg/mL BSA, pH 7.4), cocaine (10 mM Tris, 100 mM NaCl, 1 mM MgCl_2 , 0.1 mg/mL BSA, pH 7.4), and ATP (10 mM Tris, 10 mM MgCl_2 , 0.1 mg/mL BSA, pH 7.4). Reaction mixtures were placed in a thermal cycler (C1000 Touch, Bio-Rad) and incubated at 23 °C for 60 min, after which 5 μL of the enzyme(s) was added to each reaction mixture. For DIS, the enzyme concentrations used were 0.13 U/ μL Exo III, 1.5 U/ μL Exo I, or a mixture of 0.13 U/ μL Exo III + 1.5 U/ μL Exo I. For cocaine, the enzyme concentrations used were 0.4 U/ μL Exo III, 0.9 U/ μL Exo I, or a mixture of 0.4 U/ μL Exo III + 0.9 U/ μL Exo I. For ATP, the enzyme concentrations used were 0.5 U/ μL Exo III, 1.4 U/ μL Exo I, or a mixture of 0.5 U/ μL Exo III + 1.4 U/ μL Exo I. For all experiments, 5 μL of sample was collected at various time points and mixed with 10 μL of formamide loading buffer (75% formamide, 10% glycerol, 0.125% SDS, 10 mM EDTA, and 0.15% (w/v) xylene cyanol) to quench the reaction. Digestion products were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). Specifically, 3 μL of each collected sample was loaded into the wells of a 15% denaturing PAGE gel. 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 Image system (Bio-Rad).

SYBR Gold Fluorescence Experiments.

The dual-exonuclease-mediated assay was performed using various concentrations of DIS (0, 0.5, 1, 2.5, 5, 10, 15, 25, 33, 50, 75, 100, and 250 μL), cocaine (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 35, 50, 75, 125, 200, 500, and 1000 μL), and ATP (0, 0.25, 0.5, 1, 2.5, 5, 10, 15, 25, 50, 100, and 250 μL). After digestion, 50 μL of digestion products was mixed with 50 μL of 2 $\times$ SYBR Gold diluted in 10 mM Tris buffer (pH 7.4) containing 100 mM EDTA and 25% formamide (v/v). On the basis of the estimated extinction coefficient of SYBR Gold as reported in the literature ($\epsilon_{494} = 50\,000\text{ M}^{-1}\text{ cm}^{-1}$),^{34,35} the concentration of 1 $\times$ SYBR Gold is equivalent to 1.21 μL . An 80 μL amount of the mixture was loaded into the wells of a 96-well plate. Fluorescence emission spectra were recorded from 510 to 850 nm using a Tecan microplate reader (Infinite M1000 PRO, Switzerland) with a 495 nm excitation wavelength. The fluorescence intensity recorded at 545 nm was used to calculate signal gain using the equation $(F - F_0)/F_0$, where F and F_0 represent fluorescence intensity in the presence and absence of target, respectively. Calibration curves were constructed by plotting signal gains against target concentrations. Each experiment was performed in triplicate, and error bars represent the standard deviation of three measurements.

Multiplex Target Detection Using Fluorophore-Quencher-Modified Molecular Beacons.

A 1 μL amount of 25 μM COC-38 and 1 μL of 25 μM ATP-33 were added into 43 μL of reaction buffer (10 mM Tris, 100 mM NaCl, 1 mM MgCl_2 , and 0.1 mg/mL BSA, pH 7.4) containing various concentrations of either cocaine, ATP, or both in a 200 μL PCR tube. Reaction mixtures were placed in a thermal cycler and incubated at 23 $^\circ\text{C}$ for 60 min, after which 5 μL of Exo III (0.5 U/ μL) only, Exo I (1.5 U/ μL) only, or exonuclease mixture (0.5 U/ μL Exo III + 1.5 U/ μL Exo I) was added into each sample. After a 20 min reaction, 40 μL of stop solution (10 mM Tris, 12.5 mM EDTA, and 500 mM NaCl, pH 7.4) was added to quench the enzymatic reaction. Then 10 μL of molecular beacon mixture (final concentration 200 nM COC-MB + 200 nM ATP-MB) prepared in hybridization buffer (10 mM Tris buffer, 500 mM NaCl, and 1 mM MgCl_2 , pH 7.4) was introduced to quantify the digested aptamer(s). An 80 μL amount of the mixture was immediately loaded into the wells of a 96-well plate. Fluorescence emission spectra were recorded with a 495 nm excitation wavelength for FAM and with a 645 nm excitation wavelength for Cy5 using a Tecan microplate reader (excitations were performed separately). Calibration curves were generated using varying concentrations of either ATP (0, 2.5, 5, 10, 25, 50, 75, 100, 250, 350 μL) or cocaine (0, 15, 20, 25, 50, 75, 100, 250, 500, 1000 μL).

Isothermal Titration Calorimetry (ITC) Experiments.

All ITC experiments were performed in reaction buffer with a MicroCal ITC200 instrument (Malvern). The sample cell contained 20 μL aptamer, with 500 μL DIS, 500 μL cocaine, or 800 μL ATP loaded in the syringe. Each experiment consisted of 19 successive 2 μL injections after a 0.4 μL purge injection with a spacing of 180 s to a final ligand to aptamer molar ratio of 5.5:1 for the cocaine-binding aptamers and DIS-binding aptamers and 8.8:1 for the ATP-binding aptamers. All experiments were performed at 23 $^\circ\text{C}$. The raw data were first corrected based on the dilution heat of each target and then analyzed with the MicroCal analysis kit integrated into Origin 7 software with a single-site binding model for DIS and cocaine or a two-site sequential binding model for ATP.

RESULTS AND DISCUSSION

Structure Selectivity of Exo III for Target-Aptamer Complexes.

Prior work has shown that the 3'-to-5' exonuclease activity of Exo III on double-stranded DNA can be stalled by covalent^{27,28} and noncovalent²⁹ DNA-binding small molecules. For example, benzo-*a*-pyrene and benzo-*c*-phenanthrene react with the primary amine group of deoxyadenosine and deoxyguanosine bases, forming covalent DNA adducts within the helical minor groove^{27,28} that halt Exo III digestion 3–4 bases prior to the binding site.^{27,28} Similar behavior was observed with noncovalent DNA-binding small molecules, such as 4', 6-diamidino-2-phenylindole, Hoechst 33258, and distamycin A, which inhibit Exo III digestion 3–6 bases prior to the binding site.²⁹ We therefore predicted that Exo III activity could be similarly affected by aptamer–target interactions.

As proof of concept, we performed Exo III digestion using a previously reported DIS-binding aptamer (DIS-37).³¹ DIS-37 exists in a fully folded state even in the absence of DIS, due to the seven Watson–Crick base pairs (bp) in stem 1 (Figure 1A). DIS binds within the

aptamer's prefolded three-way-junction-structured target-binding domain without inducing a large conformational change in the aptamer. We performed a time course of DIS-37 Exo III digestion in the presence and absence of DIS and characterized the digestion products using denaturing PAGE, with concentrations calculated relative to a customized DNA ladder loaded in the gel. Regardless of the presence or absence of DIS, Exo III rapidly removed the first three nucleotides from the 3' end of DIS-37. In the absence of DIS, Exo III continued digestion, forming three intermediate products (33-, 32-, and 31-nt) and eventually resulting in a 30-nt major product (Figure 1B). In the presence of DIS, further Exo III digestion was greatly inhibited, resulting in accumulation of 33- and 34-nt major products (Figure 1B and 1C). These results demonstrate that Exo III digestion of DIS-37 is strongly inhibited upon the addition of target. We then synthesized the 30-, 33-, and 34-nt major products (DIS-30, DIS-33, DIS-34) and used isothermal titration calorimetry (ITC)³⁶ to investigate their binding affinity for DIS. We obtained an equilibrium dissociation constant (K_D) of $4.0 \pm 0.2 \mu\text{M}$ for DIS-34 (Supporting Information (SI) Figure S1A), which is very similar to the K_D of DIS-37 ($3.4 \pm 0.2 \mu\text{M}$, SI, Figure S1B). A 4-fold decrease in binding affinity was observed for DIS-33 ($K_D = 16.0 \pm 1.0 \mu\text{M}$, SI, Figure S1C) due to its reduced thermostability. These results suggest that the major products generated in the presence of DIS remain bound to the target. In contrast, DIS-30 resists Exo III digestion but retains no binding affinity for DIS (SI, Figure S1D), indicating this product presumably exists in a single-stranded state.³²

Inhibition of Exo III Requires Formation of the Target-Aptamer Complex.

To confirm that Exo III digestion of DIS-37 is modulated specifically by target binding, we designed a mutant aptamer with no binding affinity for DIS. This mutant (DIS-37-M) was designed by changing two nucleotides within the target-binding domain; specifically, the adenine at position 21 and the thymine at position 30 were both changed to cytosine (SI, Figure S2A). We expected that DIS-37-M would retain a three-way junction structure but with impaired target-binding affinity. In order to verify that the aptamer is fully folded, we made use of Exo I, which is able to digest single-stranded but not double-stranded DNA.³² We incubated both DIS-37 and DIS-37-M with Exo I and found that it was unable to digest either aptamer regardless of the presence or absence of DIS (SI, Figure S3). This indicated that stem 1 was fully folded in both aptamers. We then used ITC to confirm that DIS-37-M possesses greatly reduced binding affinity for DIS ($K_D > 1000 \mu\text{M}$, SI, Figure S2B). To evaluate whether the target itself inhibits Exo III activity, we performed Exo III digestion with DIS-37-M. We found that Exo III digestion of the mutant yielded the same major products (32-, 31-, and 30-nt) regardless of the presence or absence of DIS (SI, Figure S3A). Given that DIS-37-M cannot form the aptamer–target complex, these results clearly indicate that DIS itself does not impair the function of Exo III. We performed the same experiment with DIS-37 and found that DIS binding inhibited Exo III digestion, whereas the aptamer alone was readily digested by the enzyme (SI, Figure S3B). These results demonstrated that the formation of the target–aptamer complex is directly responsible for inhibiting Exo III digestion.

Mechanism of Exo III Inhibition and Development of the Dual-Exonuclease-Mediated Assay.

We experimentally observed that Exo III digestion of DIS-37 occurred via a two-phase process. First, Exo III rapidly catalyzed the digestion of the double-stranded 3' end of

DIS-37, removing three nucleotides to form a 34-nt product regardless of the presence or absence of DIS. In the absence of target, Exo III continued to digest the 34-nt product into 33-, 32-, and 31-nt products at a progressively slower rate, eventually resulting in a 30-nt major product. In the presence of target, the 34-nt product greatly inhibited Exo III digestion. To understand the mechanism of inhibition, we digested DIS-30 and DIS-34 with either Exo III or Exo I. Regardless of the presence or absence of target, DIS-30 was not digested by Exo III but was completely digested by Exo I (Figure 2A). This confirms that the 30-nt product primarily exists in a single-stranded state, even in the presence of 250 μM DIS. Both Exo III and Exo I could digest DIS-34 in the absence of target (Figure 2B), indicating that nontarget-bound DIS-34 is in equilibrium between single- and double-stranded states. In contrast, Exo III digestion of DIS-34 was greatly inhibited in the presence of DIS due to formation of the target–aptamer complex. Similarly, target-bound DIS-34 was not digested by Exo I, indicating that the aptamer was predominantly in a double-stranded state when bound to DIS (Figure 2B).

On the basis of these results, we developed a dual-exonuclease-mediated assay that utilizes synergistic digestion by Exo III and Exo I to quantify DIS concentrations. We predicted that nontarget-bound DIS-37 would first be digested by Exo III to form several products that are in equilibrium between single- and double-stranded states, which can be completely digested by Exo I. In contrast, in the presence of DIS, Exo III will remove only three nucleotides from DIS-37, with the resulting target-bound 34-nt product remaining resistant to digestion by both exonucleases. PAGE analysis of this digestion experiment confirmed that DIS-37 was completely digested in the absence of target. In the presence of target, we observed a 34-nt major product, and the concentration of this product increased with increasing concentrations of DIS until reaching saturation at 250 μM (Figure 3A and 3C). In order to determine whether higher concentrations of DIS might inhibit either exonucleases' activity, we digested the mutant aptamer, DIS-37-M, with the exonuclease mixture. The mutant was completely digested at DIS concentrations of up to 500 μM (Figure 3B and 3C), confirming that DIS itself inhibits neither Exo III nor Exo I.

Label-Free Detection of DIS Using a Dual-Exonuclease-Mediated Fluorescence Assay.

We subsequently adapted our dual-exonuclease-inhibition approach into a label-free fluorescence-based assay for the sensitive detection of DIS. Digestion of DIS-37 yielded intact oligonucleotide products in the presence of DIS, whereas the aptamer was completely degraded into mononucleotides in the absence of DIS (Figure 4A). Therefore, we selected SYBR Gold as a signal reporter, since it fluorescently stains only oligonucleotides but not mononucleotides.³³ Following a 15 min digestion of DIS-37 by the exonuclease mixture, we added a solution containing EDTA and formamide to deactivate the exonucleases and denature all major products into single-stranded structures. After adding 1 $\times$ SYBR Gold, we observed a 40-fold signal gain for a sample containing 250 μM DIS relative to the target-free sample, with only minimal background in the absence of DIS (Figure 4B). As a control, we monitored target-binding-induced fluorescence changes of DIS-37 without exonuclease treatment and observed virtually no difference in fluorescence intensity upon the addition of 250 μM DIS (Figure 4B).

We further measured the sensitivity of our assay by generating a fluorescence calibration curve using different concentrations of DIS (Figure 4C). The fluorescence signal gain increased as the DIS concentration increased (Figure 4D) with a linear range from 0 to 10 μM and a measurable detection limit³⁷ of 500 nM (Figure 4E). Importantly, our dual-exonuclease-mediated assay demonstrated robust performance in biological matrices, and we obtained a similar linear range and detection limit for DIS in 50% urine samples (Figure 4E). The quantity of 34-nt digestion product generated at different DIS concentrations was then characterized using denaturing PAGE, with concentrations calculated relative to a DIS-34 standard loaded in the gel (SI, Figure S4). We found that 173, 287, 359, 507, 667, 718, and 863 nM 34-nt product were generated in the presence of 15, 25, 33, 50, 75, 100, and 250 μM DIS, respectively. It was difficult to accurately determine the concentration of 34-nt product formed at low concentrations of DIS (0.5–10 μM). Therefore, we used 1 $\times$ SYBR Gold to create a calibration curve using DIS-34 (SI, Figure S5). On the basis of this curve, we obtained concentrations of 8.6, 11, 25, 60, and 111 nM of the 34-nt product in the presence of 0.5, 1, 2.5, 5, and 10 μM DIS, respectively.

Application of the Dual-Exonuclease-Mediated Assay to a Three-Way Junction Cocaine Aptamer.

We next demonstrated the generality of our assay with a cocaine-binding aptamer (COC-38),³⁸ which is prefolded and contains a three-way-junction-structured binding domain like DIS-37. COC-38 contains a 7-base-pair blunt-ended stem and is a preferred substrate for Exo III. We incubated COC-38 with Exo I, Exo III, or the exonuclease mixture in the absence and presence of cocaine and characterized the digestion products using PAGE (Figure 5A). Exo I alone did not digest this prefolded aptamer regardless of the presence or absence of cocaine (Figure 5A, Exo I). However, Exo III showed a target-dependent digestion profile (Figure 5A, Exo III). Specifically, we observed a 33-nt major product in the absence of target, which we believe is single stranded due to the destabilization of the stems. In the presence of cocaine, only three nucleotides were removed from COC-38 to form a 35-nt product, and further Exo III digestion was strongly inhibited (SI, Figure S6). As with DIS-34, this 35-nt product is presumably capable of retaining the target and thus remains folded. ITC experiments confirmed that this 35-nt product binds to cocaine (COC-35, $K_D = 16.9 \pm 0.7 \mu\text{M}$) (SI, Figure S7A) with a similar affinity to COC-38 ($K_D = 11.2 \pm 0.6 \mu\text{M}$) (SI, Figure S7B), whereas the 33-nt product (COC-33) has no affinity for cocaine (SI, Figure S7C). Similar to DIS-37, nontarget-bound COC-38 was completely digested by the exonuclease mixture, but both enzymes were strongly inhibited by the target-bound 35-nt product (Figure 5A, Exo M).

To confirm that enzymatic inhibition was specifically associated with formation of the target-aptamer complex, we performed exonuclease digestion with a COC-38 mutant (COC-38-M) containing a single adenine-to-guanine mutation at position 22 (Figure 5B) that eliminates its binding affinity for cocaine³⁹ (SI, Figure S7D). COC-38-M was resistant to Exo I digestion, indicating that it retains the same prefolded structure as COC-38. (Figure 5B, Exo I). The Exo III digestion profiles of COC-38-M were identical regardless of the presence or absence of cocaine (Figure 5B, Exo III), and the mutant was completely digested by the exonuclease mixture with and without cocaine (Figure 5B, Exo M). Taken

together, these results show that target binding is crucial for exonuclease inhibition. We also found that the concentration of the major 35-nt product of COC-38 increased with increasing concentrations of cocaine when the exonuclease mixture was used, while up to 800 μM cocaine did not inhibit exonuclease digestion of COC-38-M (SI, Figure S8). When we replicated our dual-exonuclease-mediated fluorescence assay for the detection of cocaine (Figure 5C and 5D), we obtained a linear range from 0 to 10 μM with a measurable detection limit of 100 nM in both buffer and 10% saliva (Figure 5E).

Application of Our Assay to a Hairpin-Structured ATP Aptamer.

We further demonstrated the generality of the dual-exonuclease-mediated assay using an ATP-binding aptamer (ATP-33)^{30,40} of which the exact structure of its target-binding domain has not been conclusively determined.^{41–43} ATP-33 is prefolded and forms a hairpin structure with a 7-bp blunt-ended stem that is resistant to Exo I digestion (Figure 6A, Exo I). Despite its structural difference relative to both DIS-37 and COC-38, ATP-33 also exhibited a target binding-dependent Exo III digestion profile. Exo III digestion produced a single-stranded 25-nt major product in the absence of ATP and a 30-nt product in the presence of ATP (SI, Figure S9) (Figure 6A, Exo III). We measured the ATP-binding affinity of the 25-nt (ATP-25) and 30-nt (ATP-30) major products as well as the parent aptamer using ITC. Given that the ATP-binding aptamer binds to two ATP molecules, we assessed the binding affinity of ATP-33 and ATP-30 in terms of the target concentration required to reach half-saturation ($K_{1/2}$).⁴⁴ We found that the binding affinity of ATP-30 ($K_{1/2} = 2.8 \pm 0.1 \mu\text{M}$, SI, Figure S10A) was much higher than that of ATP-25 ($K_D = 595 \pm 86 \mu\text{M}$, SI, Figure S10B) (K_D is used for ATP-25 as it only binds a single target).⁴⁵ As with the other two aptamers, the major product of Exo III digestion from target-bound ATP-33 essentially retained the affinity of the undigested aptamer ($K_{1/2} = 2.8 \pm 0.1 \mu\text{M}$, SI, Figure S10C). We also observed a target-dependent digestion profile with the exonuclease mixture (Figure 6A, Exo M).

To confirm that enzymatic inhibition is specifically due to ATP binding, we engineered an ATP-33 mutant (ATP-33-M) by changing guanine to adenine at position 12 and adenine to thymine at position 23 (Figure 6B). ATP-33-M possesses no binding affinity to ATP (SI, Figure S10D) but maintains the same secondary structure as ATP-33, as confirmed by inhibition of Exo I digestion (Figure 6B, Exo I). As expected, the Exo III and exonuclease mixture digestion profiles of ATP-33-M were identical regardless of the presence or absence of ATP (Figure 6B, Exo III and Exo M). We performed digestion of ATP-33 and ATP-33-M with the exonuclease mixture at various concentrations of ATP and observed that the concentration of the major 30-nt product of ATP-33 increased with increasing concentrations of target (SI, Figure S11A and S11C). Moreover, we found that ATP itself did not inhibit exonuclease activity, even in the presence of 250 μM ATP (SI, Figure S11B and S11C). Finally, we carried out our dual-exonuclease-mediated fluorescence assay using ATP-33 and achieved a linear range from 0 to 10 μM , with a detection limit of 250 nM (Figure 6C–E). These results clearly demonstrated that our exonuclease assay is broadly applicable to diverse aptamers with different secondary structures.

Multiplex Detection of Cocaine and ATP Using Fluorophore-Quencher-Modified Molecular Beacons.

Multiplex detection is a valuable tool for medical diagnostics, drug screening, and food safety as it enables detection of a multitude of analytes with only a single low-volume sample.^{46–48} Our dual-exonuclease-mediated assay can be easily expanded for simultaneous detection of multiple small-molecule analytes. The nucleotide sequences of each aptamer digestion product are known and unique for each aptamer–target pair. These aptamer digestion products can therefore serve as proxies for each individual target and can be quantified using molecular beacons that are designed to specifically hybridize with an individual digested aptamer strand. The resulting signal can be used to accurately determine the concentration of the target. As a demonstration, we employed our dual-exonuclease-mediated assay with two fluorophore–quencher-modified molecular beacons to simultaneously detect ATP and cocaine. The design of our molecular beacons is very simple, as they share the same 6-bp stem but with different complementary loop regions. The molecular beacon for ATP (ATP-MB) contains a 28-nt loop that is complementary to the 30-nt product and is modified with a 5′ Cy5 and 3′ Iowa Black RQ quencher, while the molecular beacon for cocaine (COC-MB) contains a 33-nt loop that is complementary to the 35-nt product and is modified with a 5′ FAM and 3′ Black Hole Quencher 1 (SI, Figure S12). To perform the multiplex assay, we digested an aptamer mixture of COC-38 and ATP-33 with both Exo III and Exo I in the presence or absence of cocaine and/or ATP, after which we added a mixture of molecular beacons to achieve target detection (Figure 7). In the presence of cocaine or ATP, the exonucleases generate specific aptamer digestion products (35-nt from COC-38 or 30-nt product from ATP-33, respectively) that hybridize with their corresponding molecular beacon, separating the fluorophore from the quencher and generating a single target-related fluorescence signal (Figure 7A and 7B). When both targets are present, two specific exonuclease digestion products are generated and both COC-MB and ATP-MB hybridize with their respective digestion product, resulting in two fluorescence signals (Figure 7C). In contrast, both exonucleases completely digest all aptamers in the absence of target, and no fluorescence signal is generated as the beacons remain in a closed and therefore quenched state (Figure 7D).

We confirmed that our dual-exonuclease-mediated assay performs effectively for multiplex detection of ATP and cocaine (Figure 8). After 20 min of digesting COC-38 and ATP-33 in the presence of various concentrations of cocaine and ATP (0, 25, or 100 μM), we added EDTA to stop digestion and then added a mixture of beacons (Final concentration: 200 nM COC-MB + 200 nM ATP-MB) to determine target concentrations through quantification of the aptamer digestion products. In the absence of target, minimal fluorescence signal was observed (Figure 8A). In the presence of 25 or 100 μM cocaine alone, we observed a concentration-dependent fluorescence signal at 520 nm (COC-MB; FAM) but minimal signal at 668 nm (ATP-MB; Cy5) (Figure 8B and 8C). Likewise, when ATP alone was present, we observed a concentration-dependent fluorescence signal at 668 nm, with minimal signal detectable at 520 nm (Figure 8D and 8E). In the presence of a mixture of both targets at various concentrations, we observed distinct concentration-dependent fluorescence signals at both 520 and 668 nm that corresponded to the presence of cocaine and ATP, respectively (Figure 8F–I). Notably, the presence of one target did not affect the

fluorescence intensity of the other, as the signals generated in the presence of cocaine and ATP were identical regardless of whether the targets were present in a mixture or alone. There was no cross-talk between the molecular beacons since each fluorophore was individually excited at their respective excitation wavelength. At 495 nm, only FAM emits at 520 nm, and we collected the spectra up to 650 nm. Cy5 may become weakly excited at 495 nm, but the emitted light (with a peak at 668 nm) does not overlap with the spectrum of FAM. Conversely, with excitation at 645 nm, only Cy5 emits at 668 nm, as FAM cannot be excited at this wavelength. These results demonstrated the capability of our dual-exonuclease-mediated assay to rapidly achieve simultaneous detection of multiple small-molecule targets in a single sample. We then generated calibration curves for our multiplex assay and obtained a linear range from 0 to 100 μM with a measurable detection limit of 15 μM for cocaine (SI, Figure S13A) and a linear range from 0 to 75 μM with a measurable detection limit of 5 μM for ATP (SI, Figure S13B).

To determine the effectiveness of using the exonuclease mixture relative to each enzyme alone, we performed a series of control experiments with cocaine as the target. Specifically, we performed Exo III, Exo I, and dual-exonuclease digestion of a mixture of COC-38 and ATP-33 with no, either, or both cocaine and ATP at a concentration 100 μM each and then added COC-MB (SI, Figure S14). Using Exo III alone, we observed a large background signal in the absence of cocaine and a very small signal gain in the presence of cocaine (SI, Figure S14A). This large background arises from the leftover single-stranded digestion products of nontarget-bound aptamers hybridizing with COC-MB to produce a nonspecific signal. Performing the assay with Exo I alone yielded a low fluorescence signal regardless of the presence or absence of target. This was expected since Exo I cannot digest the duplexed stems of fully folded aptamers, and the molecular beacon can only hybridize with a small fraction of such undigested aptamers in the absence of target. The addition of cocaine stabilizes the folded aptamers, which makes it more difficult to open the molecular beacon, yielding slightly reduced fluorescence (SI, Figure S14B). When a mixture of Exo III and Exo I was employed, we observed an extremely low background in the absence of the cocaine due to complete digestion of the aptamers, while a clear and large target-related signal gain was obtained in the presence of target (SI, Figure S14C). These results clearly demonstrate the necessity of using the exonuclease mixture.

CONCLUSION

The binding of small molecules to DNA has been shown to inhibit exonuclease activity.^{27–30} In this work, we found that the binding of small-molecule targets to their respective aptamers can also inhibit exonuclease digestion, and to the best of our knowledge, this is the first work to take advantage of this fact to develop a small-molecule detection assay based on the quantification of aptamer digestion products. We first developed a simple dual-exonuclease-mediated assay that can be generally implemented with unmodified, prefolded aptamers for small-molecule detection. In the absence of target, Exo III digests the aptamer to produce a single-stranded product, which is then completely digested by Exo I. Aptamer-target binding inhibits Exo III digestion four nucleotides prior to the binding site, leaving behind a double-stranded, target-bound product that remains invulnerable to Exo I digestion. We then used SYBR Gold, a DNA-binding dye that stains DNA in a sequence-independent

manner, to quantify the remaining intact aptamer fragments, which reflect the concentration of target present in the sample. This assay allows for rapid, label-free, simple, and sensitive detection of small-molecule targets. We finally demonstrated the generality of our dual-exonuclease-mediated approach and fluorescence assay using aptamers with various secondary structures, including three-way junction and hairpin structures. Importantly, our assay also demonstrated excellent performance in complex biological samples such as saliva and urine.

Our method provides many advantages over existing aptamer-based assays. First, no sequence engineering or aptamer truncation is required, and prefolded aptamers are used instead. Second, target detection is achieved via the quantification of aptamer digestion products instead of target-displaced cDNA, which is preferable given that the use of cDNA usually reduces assay sensitivity.^{24,25} Moreover, our assay showed much higher sensitivity compared with previously reported nuclease-assisted aptamer-based assays (SI, Table S1). For example, we achieved a 50-fold lower limit of detection for cocaine than an Exo I-based assay reported by Lou, which used structure-switching aptamers for target detection.⁴⁹ This can be attributed to two factors. First, we employed a prefolded aptamer which has a higher target-binding affinity than the structure-switching aptamer used by Lou. Second, their assay suffered from high background because Exo I could not completely digest all nontarget-bound aptamers. In our assay, the synergistic activity of Exo III and Exo I completely removed all nontarget-bound aptamers, resulting in minimal background and a robust signal-to-noise ratio.

Since each aptamer–target pair generates a digestion product possessing a unique sequence, we were able to achieve multiplex small-molecule detection within 25 min in a single sample. This was done by quantifying the digestion product of each aptamer–target pair with a complementary fluorophore–quencher-modified molecular beacon. We demonstrated one-pot detection of ATP and cocaine, where each target could be individually or simultaneously detected via the corresponding molecular beacon(s). We observed no cross talk between either aptamer-molecular beacon pair in binary target mixtures, allowing for rapid and robust simultaneous detection. On the basis of these successful results, we believe our assay can be applied for various applications where simultaneous detection of small-molecule targets is key, such as medical diagnostics, drug screening, food safety, environmental monitoring, and biodefense.^{46–48} Importantly, since the detection of small-molecule targets in our assay is achieved via quantification of DNA, one can readily envision adopting different DNA signal-amplification strategies such as enzyme-assisted target recycling^{50,51} and rolling circle amplification^{52,53} to achieve subnanomolar small-molecule detection. Although our assay in its current format is not well suited for on-site detection given the requirement of multiple steps and a fluorimeter, we believe that these limitations can be overcome with the use of a multichannel microfluidic device combined with a colorimetric signal reporter (e.g., gold nanoparticles).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Time course of Exo III digestion of a DIS-binding aptamer (DIS-37) in the absence or presence of DIS. (A) Oligonucleotide structure of DIS-37. (B) PAGE analysis of DIS-37 digestion products over 60 min with or without 250 μ L DIS. (C) Schematic of Exo III-mediated digestion of nontarget-bound (left) and target-bound (right) DIS-37.

Figure 2.

PAGE analysis of DIS-30 and DIS-34 after 15 min of exonuclease digestion in the absence and presence of 250 μ M DIS. Oligonucleotide structure and PAGE analysis of exonuclease digestion products of (A) DIS-30 and (B) DIS-34.

Figure 3.

Exonuclease digestion of (A) DIS-37 and (B) its mutant (DIS-37-M) at increasing concentrations of DIS after 15 min. Aptamers were digested with a mixture of Exo III and Exo I, and PAGE analysis was used to characterize the digestion products. (C) Calibration curves of the calculated concentration of all digestion products of DIS-37 and DIS-37-M (based on DNA ladder intensity) versus DIS concentration.

Figure 4.

DIS detection using our label-free, aptamer-based dual-exonuclease-mediated fluorescence assay. (A) Scheme of the DIS-detecting assay using SYBR Gold as a signal reporter. (B) Fluorescence spectra of SYBR Gold for exonuclease-mixture-treated (red and black lines) and untreated (blue line) DIS-37 in the presence or absence of 250 μM DIS. (C) Fluorescence spectra of the samples generated at various DIS concentrations. (D) Calibration curve derived from the fluorescence spectra. (E) Linear range of the assay in buffer as well as 50% urine.

Figure 5.

PAGE analysis of exonuclease digestion products of COC-38 and COC-38-M with and without 250 μM cocaine after 25 min of reaction and performance of the cocaine-detecting dual-exonuclease-mediated fluorescence assay. Oligonucleotide structure of (A) COC-38 and (B) COC-38-M and PAGE analysis of their digestion products generated by Exo III, Exo I, or a mixture of both. Mutated nucleotides are marked in red. (C) Fluorescence spectra of the samples generated at various cocaine concentrations. (D) Calibration curve derived from the fluorescence spectra. (E) Linear range of the assay in buffer as well as 10% saliva.

Figure 6.

PAGE analysis of exonuclease digestion products of ATP-33 and ATP-33-M with and without 250 μ M ATP after 20 min of digestion and performance of the dual-exonuclease-mediated fluorescence assay for ATP detection. Oligonucleotide structure of (A) ATP-33 and (B) ATP-33-M and PAGE analysis of their digestion products generated by Exo III, Exo I, or a mixture of both. Mutated nucleotides are marked in red. (C) Fluorescence spectra of the samples generated at various ATP concentrations. (D) Calibration curve derived from the fluorescence spectra. (E) Linear range of the assay in buffer.

Figure 7.

Scheme of dual-exonuclease-mediated, molecular beacon-based multiplexed detection of (A) cocaine alone, (B) ATP alone, (C) both cocaine and ATP, and (D) neither target. Fluorescence spectra of FAM (green) and Cy5 (red) generated in the presence or absence of 100 μ M cocaine and/or ATP.

Figure 8.

Fluorescence spectra of COC-MB (green, emission peak at 520 nm) and ATP-MB (red, emission peak at 668 nm) for exonuclease mixture-treated samples with no, either, or both cocaine and ATP at concentrations of 0, 25, or 100 μM .