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High-Affinity Aptamers for *In Vitro* and *In Vivo* Cocaine Sensing

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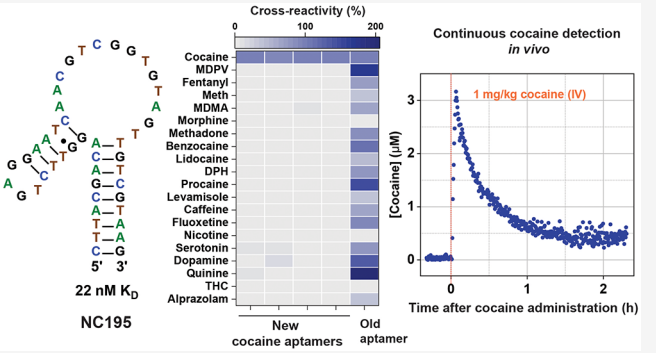
ABSTRACT: The ability to quantify cocaine in biological fluids is crucial for both the diagnosis of intoxication and overdose in the clinic as well as investigation of the drug's pharmacological and toxicological effects in the laboratory. To this end, we have performed high-stringency *in vitro* selection to generate DNA aptamers that bind cocaine with nanomolar affinity and clinically relevant specificity, thus representing a dramatic improvement over the current-generation, micromolar-affinity, low-specificity cocaine aptamers. Using these novel aptamers, we then developed two sensors for cocaine detection. The first, an *in vitro* fluorescent sensor, successfully detects cocaine at clinically relevant levels in 50% human serum without responding significantly to other drugs of abuse, endogenous substances, or a diverse range of therapeutic agents. The second, an electrochemical aptamer-based sensor, supports the real-time, seconds-resolved measurement of cocaine concentrations *in vivo* in the circulation of live animals. We believe the aptamers and sensors developed here could prove valuable for both point-of-care and on-site clinical cocaine detection as well as fundamental studies of cocaine neuropharmacology.



INTRODUCTION

Given the ubiquity of cocaine abuse and its enormous impact on public health, the ability to detect this drug rapidly, conveniently, and sensitively in biological matrices would be extremely valuable. Such a capability would improve the speed and accuracy with which cocaine overdose is detected in clinical contexts and cocaine intoxication is detected in roadside settings.[1−3] Similarly, the ability to monitor cocaine concentrations with high frequency and in real time could revolutionize laboratory investigations of the drug's pharmacokinetics, including improved understanding of molecular transport between body compartments, and pharmacodynamics by enabling concentration-based (as opposed to conventional dose response) understanding of neurochemical and behavioral responses. Unfortunately, existing approaches for detecting cocaine in clinical and laboratory settings are ill-suited for these tasks. Rapid clinical detection, for example, relies on immunoassays,[4,5] which generally produce qualitative readouts that reduce their value in formally diagnosing cocaine overdose (which is a *quantitative* phenomenon), or cumbersome laboratory-based assays that struggle to return an answer in a clinically relevant time frame. And in biomedical research, cocaine pharmacokinetics in humans has historically been monitored using periodic sampling (via blood draws or microdialysis) followed by postfacto analysis using mass spectrometry.[6] The time resolution of these approaches—other than one rather heroic effort that pushed microdialysis sampling to 2 min[7]—is in the tens of minutes resolution[8,9]—which is far slower than the tens-of-seconds time scales relevant to the absorption, distribution, and metabolism of cocaine, and the onset of its physiological effects.[2]

Motivated by the unmet need for improved methods of cocaine measurement, here we have isolated several new high-performance, cocaine-binding DNA aptamers and used them to develop sensors for the rapid, highly sensitive, and specific detection of this drug *in vitro* as well as its high-temporal-resolution measurement *in vivo*. Aptamers are short nucleic acid receptors isolated from randomized oligonucleotide libraries via the systematic evolution of ligands by exponential enrichment (SELEX) procedure to bind specific targets with high affinity.[10,11] They have several favorable attributes in the context of sensor development, including low cost of production, low batch variability, and amenability to chemical modification with signaling tags.[12,13] Aptamers have been adapted into a number of sensing platforms that are able to detect analytes directly in complex milieus with minimal or no preparation. For example, aptamer strand-displacement fluorescence sensors can detect analytes with high sensitivity in biological samples, such as pharmaceuticals and metabolites,

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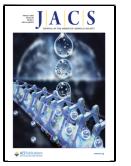

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with just a single mix-and-read step.[14−16] Likewise, electrochemical aptamer-based (EAB) sensors[17] can enable high-frequency, real-time measurement of analytes in the bloodstream, subcutaneous space, and brains of live animals.[18−21] The sensitivity and specificity of these sensors, however, are limited by the binding properties of the aptamers they employ. For example, the single existing (and widely employed) DNA aptamer against cocaine has a modest, ∼5 μM dissociation constant (K_D)[22] and is thus insufficient for monitoring cocaine at clinically relevant concentrations in biological fluids (relevant concentration range: 10−1000 nM).[23,24] In response, we here have utilized a high-stringency, library-immobilized SELEX[25,26] workflow incorporating a rigorous counter-SELEX component[27] to isolate high-affinity DNA aptamers specific against this important target. We then adapted one of the high-performance aptamers into a fluorescent sensor that demonstrated the successful detection of cocaine in serum at clinically relevant concentrations in biological fluids *in vitro*. In parallel, we developed an EAB sensor supporting the high-frequency, real-time monitoring of cocaine *in situ* in the bodies of live rats. The cocaine aptamers and sensors developed here should prove useful for detecting this drug in a wide range of settings and applications.

MATERIALS AND METHODS

Oligonucleotides. All DNA oligonucleotides were purchased from Integrated DNA Technologies (Supporting Information (SI), Table S1). The DNA used for SELEX and fluorescent sensors was purchased as HPLC-purified. All other oligonucleotides were purchased as standard desalt quality. Thiol-and-methylene-blue-modified DNA sequences were dual-HPLC-purified by the manufacturer. All oligonucleotides were dissolved in molecular biology-grade water and their concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

Materials and Reagents. Molecular biology-grade water and black 384-well flat bottom microplates were purchased from Corning. Exonuclease I (*E. coli*), Exonuclease III (*E. coli*), and T5 Exonuclease (*E. coli*) were purchased from New England Biolabs. Bovine serum albumin, Tris base, Tris HCl, 5 M NaCl solution, 1 M $MgCl_2$ solution, potassium chloride, sodium hydroxide, sulfuric acid, 6-mercapto-1-hexanol, tris(2-carboxyethyl) phosphine, Triton X-100, lidocaine HCl, diphenhydramine HCl, procaine HCl, benzocaine, ibuprofen sodium, acetaminophen, caffeine, quinine hemisulfate hydrate, serotonin HCl, xylene cyanol, acrylamide, bis-acrylamide, and 10 kDa molecular weight cutoff filters (0.5 mL capacity) were purchased from Sigma-Aldrich. Levamisole HCl was purchased from MP Biomedicals. Scopolamine hydrobromide trihydrate was purchased from Acros Organics. Nicotine, mephedrone HCl, methylenedioxypyrovalerone (MDPV) HCl, methylphenidate HCl, fentanyl HCl, (+)-methamphetamine HCl, methylenedioxy-methamphetamine (MDMA) HCl, morphine sulfate, oxycodone HCl, fluoxetine HCl, and methadone HCl were purchased from Cayman Chemicals. Cocaine HCl was purchased from Cayman Chemicals and Sigma-Aldrich. Formamide and human serum (normal pool) were purchased from Fisher Scientific. Gravity columns (800 μL bed volume) were purchased from Bio-Rad. Streptavidin-coated agarose resin (capacity: 1−3 mg biotinylated BSA/mL resin) and SYBR Gold were purchased from Thermo Fisher Scientific. GoTaq Hot Start Colorless Master Mix was purchased from Promega. PCR purification kits were purchased from Qiagen. 20× PBS was purchased from Santa Cruz Biotechnologies. Bovine blood was purchased from Hemostat Laboratories. Gold wire (75 μm diameter and 64 μm insulation thickness) was purchased from A-M systems. PTFE tubing (HS Sub-Lite-Wall, 0.02 in., black opaque) was purchased from Zeus. A 60/40 lead−selenium solder was purchased from Digikey. Platinum counter electrodes, Ag/AgCl (3 M KCl) reference electrodes, and gold-plated pin connectors were obtained from CH Instruments. For *in vivo* sensor fabrication, gold wire (0.2 mm diameter × 10 cm length; 99.9% purity), platinum wire (0.125 mm diameter × 10 cm length; 99.95% purity), and silver wire (0.125 mm diameter × 10 cm length; 99.99% purity) were purchased from A-M systems.

Buffers. The following buffers were employed in this work:

1) Selection buffer: 20 mM Tris-HCl (pH 7.4 at 25 °C), 140 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$
2) Physiological buffer: 20 mM Tris-HCl (pH 7.4 at 37 °C), 140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$

SELEX Procedure. Library-immobilized SELEX[25] was performed on a previously enriched pool selected from a 73-nucleotide (nt) stem-loop structured randomized DNA library using cocaine as a target.[28] Specific selection conditions are provided in SI, Table S2. First, the oligonucleotide pool was hybridized with a biotinylated complementary DNA (cDNA-bio) in selection buffer by heating at 95 °C for 10 min and then gradually cooling over 25 min in a room temperature water bath. After this, the library-cDNA complex was loaded into a microgravity column containing 250 μL streptavidin-agarose resin prewashed with selection buffer. Following library immobilization, the resin was washed several times with 250 μL aliquots of selection buffer to remove sequences that failed to hybridize strongly to the cDNA. Thereafter, counter-SELEX was performed as described in SI, Table S2 followed by more washes with selection buffer. Finally, the library was challenged with three aliquots of 250 μL cocaine, and the eluent was collected and purified with molecular biology-grade water using a 10 kDa molecular weight cutoff filter. The resulting sequences were PCR amplified using the GoTaq Hot Start Colorless Master Mix with 1 μM forward primer (FP) and 1 μM biotinylated reverse primer (RP-bio) using a BioRad C1000 thermal cycler with the following conditions: 2 min at 95 °C; 11 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 45 s; 5 min at 72 °C. The amplicons were converted to single-stranded DNA using streptavidin-agarose resin and 0.2 M NaOH as a denaturant. The affinity of the final-round SELEX pool for cocaine was determined using a gel elution assay.[29]

High-Throughput Sequencing (HTS) and Bioinformatic Analysis. SELEX pools were sequenced by Azenta Life Sciences using an Illumina sequencing platform. Prior to sample submission, the pools were PCR amplified with forward and reverse primers containing partial Illumina adapters (HTS-FP and HTS-RP). The amplicons were purified using a PCR purification kit (Qiagen) and then with 10 mM Tris buffer (pH 7.4) using a 10 kDa filter. Amplicons were submitted as 25 μL samples containing 20 ng/mL of double-stranded DNA. HTS data were received from Azenta as fastq files and processed using cutadapt[30] to trim primers and then FASTAptamer[31] to align, count, and cluster sequences.

Exonuclease Digestion Assays. Digestion experiments were performed at 25 or 37 °C using a dry bath incubator with sample volumes of 50 μL. First, aptamers (final concentration of 0.5 μM) were diluted in Tris buffer (pH 7.4 at the respective reaction temperature, final concentration of 20 mM), heated to 95 °C for 5 min, and immediately cooled on ice for 3 min to promote intramolecular hybridization. Next, 1 μL salt solution (final concentration for selection buffer: 140 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$; final concentration for physiological buffer: 140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$) and 0.5 μL bovine serum albumin (BSA; final concentration: 0.1 mg/mL) was added into 3.5 μL aptamer solution. Then, 20 μL of either buffer, interferent, or cocaine was added, and the solution was incubated at the appropriate reaction temperature for 1 h. Afterward, exonuclease solutions were prepared in buffer containing BSA (final concentration: 0.1 mg/mL). Binding profile determination experiments (which used T5 Exo and Exo I or Exo III and Exo I) were performed in selection buffer, while Exo I-only assays were performed in physiological buffer. Depending on the experiment, final concentrations were 0.2 U/μL T5 Exo and 0.015 U/μL Exo I; 0.025 U/μL Exo III and 0.05 U/μL Exo I; or 0.05 U/μL Exo I alone. 25 μL of exonuclease solution was added to the aptamer solution to initiate digestion. For microplate assays, a 5 μL aliquot of the sample was taken at various time-points and mixed with 30 μL of

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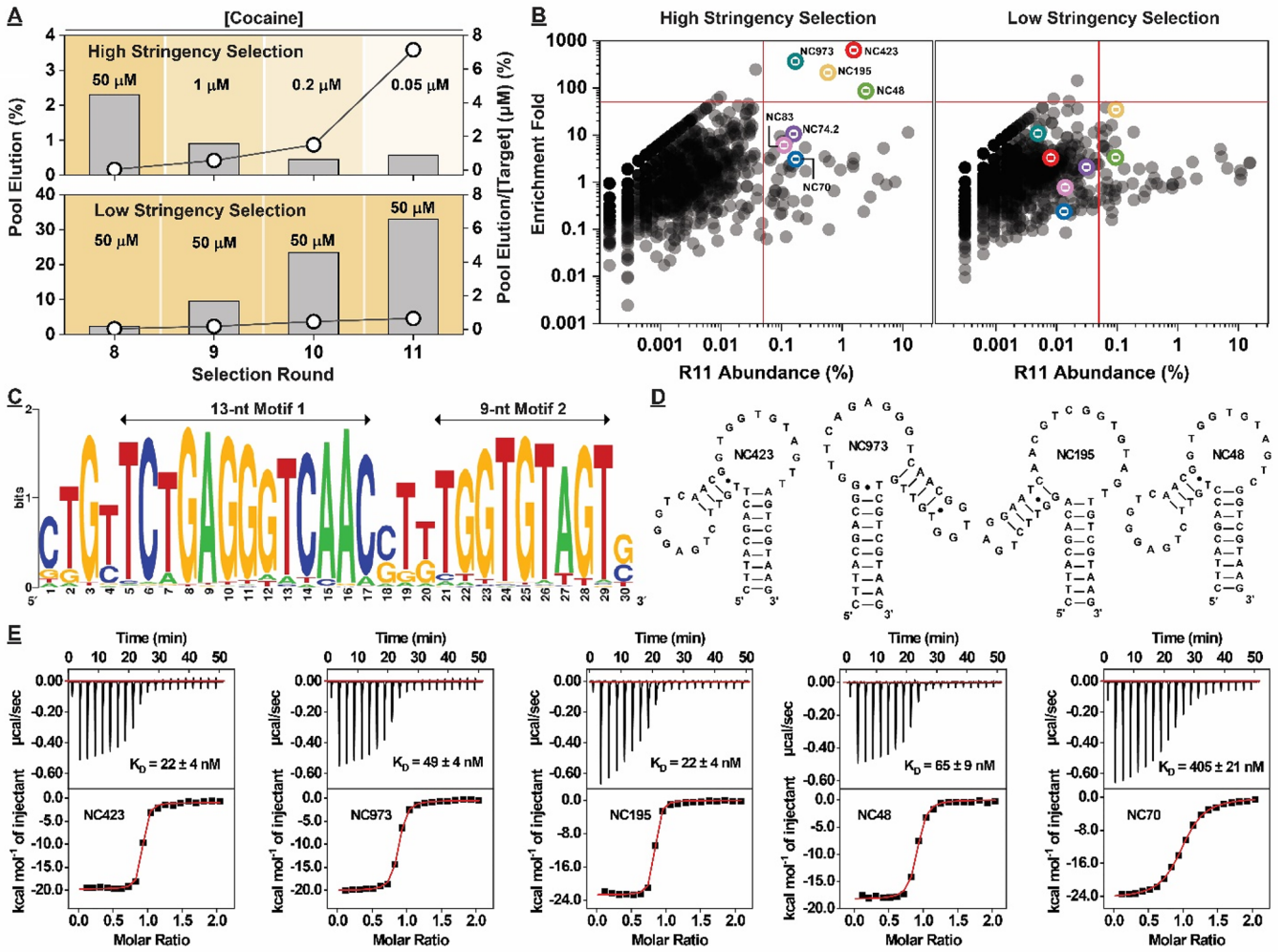

Figure 1. Shown is the isolation and characterization of new DNA aptamers binding cocaine with exceptional affinity and specificity. (**A**) The proportion of the pool eluted by cocaine (bars) in rounds 8−11 of the low- and high-stringency SELEX trials. Target concentrations are shown and indicated by the color gradient. The individual data points and line indicate pool elution divided by the target concentration. (**B**) High-throughput sequencing data for the high- (left) and low- (right) stringency selection trials. Enrichment fold between rounds 8 and 11 is plotted against the abundance of each sequence in round 11. Red lines indicate quality thresholds for high-affinity aptamer candidates. These candidates and three control sequences are color-coded and labeled in the lefthand panel. (**C**) Sequence logo of the newly isolated high-affinity cocaine aptamer family. (**D**) Secondary structures of high-affinity aptamers NC423, NC973, NC195, and NC48. (**E**) Isothermal titration calorimetry data for determining the cocaine-binding affinity of these aptamers and the control sequence NC70.

quenching solution (final concentration: 10 mM Tris, pH 7.4, 1× SYBR Gold, 21 mM EDTA, and 12.5% formamide) loaded in a black 384-well flat bottom microplate. Fluorescence was measured using a Tecan M1000 Pro microplate reader with an excitation wavelength of 495 nm and emission of 537 nm. Each sample was measured 10 times, and the average of these measurements was used for analysis. Resistance values were calculated as described previously.[32] For PAGE analysis, 5 μL aliquots of the samples were diluted in 10 μL of loading buffer (final concentration: 0.125% (w/v) xylene cyanol FF, 10% glycerol (v/v), 0.125% (w/v) SDS, 10 mM EDTA, 75% formamide). The samples were run using 15% acrylamide gels in 0.5× TBE initially at 6 V/cm for 0.5 h and then at 25 V/cm for 4 h. Gels were subsequently stained with 1× SYBR Gold and imaged by using a BioRad Gel Imaging Station.

Isothermal Titration Calorimetry (ITC) Experiments. All ITC experiments were performed in selection buffer or physiological buffer with a MicroCal PEAQ-ITC instrument (Malvern) at either 23 or 37 °C. Refer to SI, Table S3 for specific conditions for each experiment. In general, aptamers were prepared in Tris buffer, heated at 95 °C for 5 min, and immediately cooled on ice for 3 min. Salts were then added to reach the appropriate final concentrations, and 300 μL of the aptamer solution was loaded into the cell. The syringe was loaded with cocaine dissolved in the same buffer. Each ITC experiment consisted of an initial purge injection of 0.4 μL and 19 successive injections of 2 μL with a spacing of 180 s between injections. The raw data were first corrected for the dilution heat of the ligand and then analyzed with the MicroCal analysis kit and fitted with a single-site binding model.

***In Vitro* Cocaine Measurements Using the Strand-Displacement Fluorescence Sensor.** This experiment was performed as described previously[15] at room temperature with a sample volume of 80 μL. The sensor utilizes NC195 labeled with 5′ Cy5 (NC195-Cy5) and a 3′ Iowa Black RQ-labeled cDNA (cDNA13Q). First, using a fixed concentration of 50 nM NC195-Cy5, the concentration of cDNA13Q was optimized to achieve >90% quenching by mixing the aptamer with various concentrations of cDNA. To do so, 39 μL of aptamer and 39 μL of cDNA dissolved in selection buffer were mixed together and subsequently heated to 95 °C for 5 min and then cooled to room temperature gradually using a water bath over 25 min. Thereafter, 2 μL of Triton-X100 was added to the samples (final concentration: 0.005% by volume). Finally, 75 μL of each sample was loaded into a black 384-well flat bottom microplate, and their fluorescence was measured using a Tecan microplate reader with excitation at 650 nm and emission at 670 nm. The optimal

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concentration of cDNA13Q was 125 nM. To detect cocaine, NC195-Cy5 and cDNA13Q (final concentrations: 50 and 125 nM, respectively) were dissolved in selection buffer, heated to 95 °C for 5 min, and cooled in a water bath for 25 min. Then, 40 μL of aptamer-cDNA solution was mixed with 40 μL of various concentrations of cocaine in buffer or human serum. After 30 min of incubation to allow the aptamer and target to bind, 75 μL of the sample was loaded into a black 384-well flat bottom microplate, and fluorescence was measured using the same excitation and emission wavelengths. Fluorescence recovery was calculated using the equation F/F_0, where F and F_0 are the fluorescences of the solution in the absence and presence of cocaine, respectively. To construct calibration curves, fluorescence recovery of each sample was plotted against the concentration of cocaine. To evaluate sensor specificity, the same procedure was followed, except the 40 μL aptamer-cDNA solution was mixed with 40 μL of either cocaine (final concentration: 0.1 or 1 μM) or interferent (final concentration: 100 μM, except for THC, AB-FUBINACA, and UR-144, which were 5 μM in selection buffer containing 5% (v/v) DMSO).

***In Vitro* Cocaine Measurements Using EAB Sensors.** A detailed protocol of EAB sensor preparation using NC195−36 and the determination of its calibration curve in whole bovine blood is provided in the Supporting Information. In brief, we fabricated EAB sensors using established protocols[18,20,33] that include depositing 5′ thiol-modified, 3′ methylene-blue-modified NC195−36 (NC195−36-MB) or vancomycin aptamer (Vanc-MB) onto a gold wire working electrode. The aptamer-modified working electrode, platinum counter electrode, and Ag/AgCl (3 M KCl) reference electrode were used in a standard three-electrode setup. We interrogated the sensors using square-wave voltammetry (SWV) and calculated the kinetic-drift measurement (KDM) signal[19] using a 200 and 20 Hz frequency pair. For calibration, we placed the sensor into undiluted whole bovine blood in a shot glass, which was maintained at 37 °C in a water bath. This was titrated with cocaine, and SWV measurements were performed after 3 min of incubation. KDM signals were plotted against cocaine concentration to obtain a Langmuir-isotherm calibration curve, which was used for *in vivo* studies to determine cocaine concentrations based on observed KDM signal.

***In Vivo* Cocaine Measurements Using EAB Sensors.** Our *in vivo* protocols are described in detail in the SI. In brief, we fabricated an intravenous sensor using previously established methods.[18,20,34] NC195−36-MB or Vanc-MB was attached to a gold working electrode, which was bundled with a platinum wire counter electrode and a silver wire reference electrode, fed through a 20-gauge catheter, and then emplaced into the right jugular vein of an anesthetized rat. A silastic catheter was placed into the left jugular vein for drug delivery. The sensor was then interrogated using SWV using frequencies of 200 and 20 Hz. We collected a >20 min baseline before challenging the animal with intravenous cocaine HCl (5 mM, 1 mg/kg IV over a period of 3 min) using a motorized syringe pump. The resulting output was drift corrected using KDM[19] and converted into concentration using the calibration curve described above.

RESULTS

Selection of New, High-Performance DNA Aptamers against Cocaine. In the course of developing a new SELEX methodology, we recently performed 11 rounds of standard library-immobilized SELEX to pre-enrich an oligonucleotide pool that binds to cocaine.[28] The pool was derived from a 73-nucleotide (nt) stem-loop structured library containing a randomized 30-nt putative binding domain and flanking constant regions for PCR amplification. For the first two rounds, we used 100 μM cocaine and then lowered cocaine concentrations to 75 μM in the third round and finally 50 μM for the remaining selection rounds (SI, Figure S1). We also performed counter-SELEX to remove oligonucleotides that bind to a variety of interferents, including other illicit drugs, common street-drug adulterants, and endogenous compounds (SI, Table S2). After sequencing and affinity characterization of aptamers from this pool, we determined that the aptamers had K_Ds in the submicromolar regime.[28] Hereafter, we refer to this as the "low-stringency trial" of SELEX. Although this represents a clear improvement in affinity relative to the widely used MNS4.1 cocaine-binding aptamer first described by Stojanovic and colleagues,[22] it remains insufficient for detecting cocaine at physiologically relevant concentrations in biological matrices, which can be as low as 10 nM.[9]

To obtain aptamers with higher affinity, we performed three rounds of selection starting from the round 8 pool of the low-stringency trial using a higher-stringency selection strategy. This entailed decreasing the concentration of cocaine 1000-fold over the course of three rounds of selection, such that only aptamers with low nanomolar affinity would survive this high-stringency trial of SELEX (Figure 1A, top). To ascertain the degree of aptamer enrichment, in each round, we monitored the quantity of pool eluted by buffer alone (i.e., background elution level) and compared it with that eluted by cocaine. We did so by collecting aliquots of all eluents during SELEX and performing polyacrylamide gel electrophoresis to obtain a quantitative measure of library elution. In the ninth round, we observed pool elution by cocaine was 4-fold above background levels, indicating enrichment of target binding sequences, despite using 1 μM cocaine, which represents a 50-fold reduction in target concentration relative to round 8 (SI, Table S2). In the next round, we reduced the concentration of cocaine to 0.2 μM. Despite this, pool elution by the target remained 2−3-fold above background levels, indicating that high-affinity aptamers continued to be enriched. We performed our final round of selection using 50 nM cocaine, which is to our knowledge the lowest target concentration ever utilized for any library-immobilized SELEX. Nevertheless, the cocaine-eluted pool still remained 2−3-fold above background levels (SI, Figure S2). Following the completion of this selection, we measured the affinity of the resulting pool using a gel elution assay.[29] This produced a slightly biphasic binding curve with a K_D of 1 and 98 μM (SI, Figure S3). This contrasts noticeably with our low-stringency trial (Figure 1A, bottom), the round 11 pool of which exhibited biphasic binding with a K_D of 4 and >1000 μM (SI, Figure S4).

Our high-stringency selection strategy successfully removed most weak binders and enriched high-affinity aptamers. We subjected the initial round 8 pool and the rounds 9−11 pools from the high-stringency trial to high-throughput sequencing, obtaining 100 000−200 000 reads for each pool. The proportion of unique sequences in the round 8 pool was ∼35%. This dropped to 13% in round 9 but then increased to 14% in round 10 and 18% in round 11 (SI, Figure S5). The increase in the proportion of unique sequences in the pools as rounds progressed was unusual, as pool diversity typically tends to decrease as more rounds are performed.[35] One possible explanation for this trend is that as target concentration was lowered every round, fewer binding sequences were being eluted by target relative to the large variety of nonfunctional background sequences that spontaneously fall off during elution, thereby artificially increasing pool diversity. The high-throughput sequencing data highlight the importance of increasing selection stringency only during later rounds, in which the population of high-affinity binders is sufficiently abundant such that the pool avoids being over competed by nonbinding sequences during SELEX.

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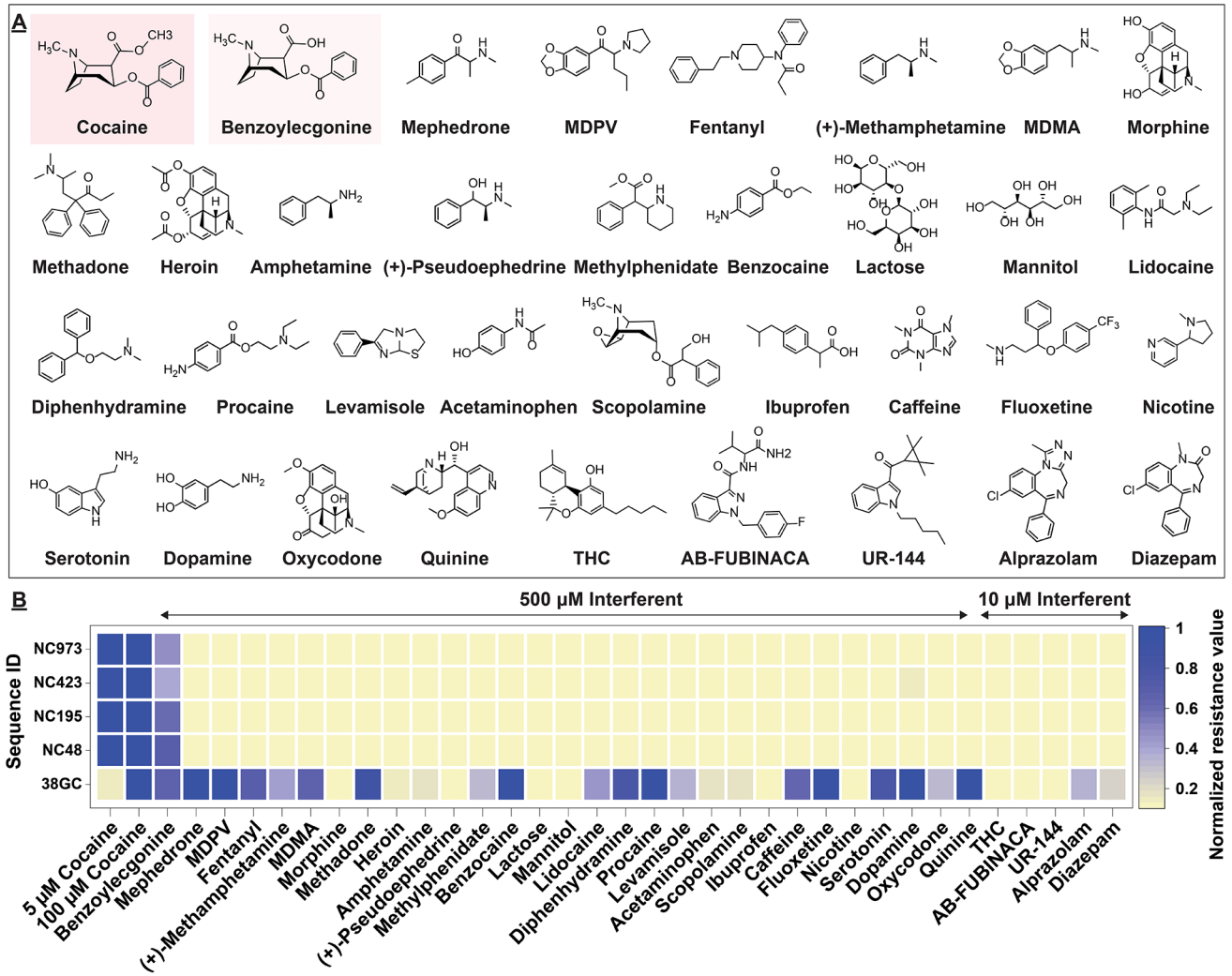

Figure 2. Exceptional specificity of new cocaine aptamers determined by an exonuclease digestion fluorescence assay. (**A**) The chemical structures of ligands (target and interferents) tested in the assay. (**B**) Heat map indicating the normalized resistance value for each aptamer against ligands. The yellow-to-blue color gradient represents increasing resistance to exonuclease digestion and hence higher aptamer-ligand binding affinity. Cocaine was employed at concentrations of 5 and 100 μM. The interferents were employed at a concentration of 500 μM, except for THC, AB-FUBINACA, UR-144, alprazolam, and diazepam, for which concentrations of 10 μM were employed due to solubility limitations.

To identify aptamer candidates for further characterization, we assessed the change in population abundance (e.g., enrichment fold) between rounds 8 and 11, with the assumption that sequences with high growth rates were preferentially enriched by the high-stringency selection process. We chose aptamer candidates that exhibited an enrichment fold >50 as well as an overall abundance >0.05% among sequences in the final round 11 pool. In the high-stringency trial, we identified four sequences—NC48, NC423, NC195, and NC973—which exhibited abundance of 0.2−2% and enrichment of 90−700-fold (Figure 1B, left); however, none of these sequences was identified in the low-stringency trial using these metrics (Figure 1B, right). We also assessed enrichment fold of these sequences between Rounds 11 and 9 and Rounds 11 and 10 and observed decreased enrichment fold as the distance between the rounds decreased (SI, Figure S6). Interestingly, NC48, NC423, NC195, and NC973 are highly related, as clustering analysis revealed they originate from a family containing two primary motifs: a 13-nt motif and a 9-nt GT-rich motif connected via a 3−4-nt linker (Figure 1C). NUPACK analysis[36] revealed these aptamers may contain a stem-bulge-stem structure (Figure 1D). Notably, when comparing the high-throughput sequencing data between both trials, these four candidate sequences were essentially indistinguishable from other sequences in the low-stringency trial in terms of their abundance and enrichment fold (Figure 1B, right). However, in the high-stringency trial, these sequences are clearly distinguishable from the rest of the population, with both high abundance and enrichment fold (Figure 1B, left). This demonstrated that higher-stringency conditions were required to effectively enrich and identify these high-affinity sequences. The largest growth in abundance of NC48, NC423, NC195, and NC973 occurred between rounds 8 and 9, with this growth decelerating in subsequent rounds (SI, Figure S7). Interestingly, the original cocaine aptamer MNS4.1 was not identified in the HTS data set, although there were six sequences with no more than 22 identical nucleotides relative to this aptamer (SI, Figure S8). Since we utilized numerous countertargets that bind to MNS4.1 (e.g., lidocaine, diphenhydramine, procaine, levami-

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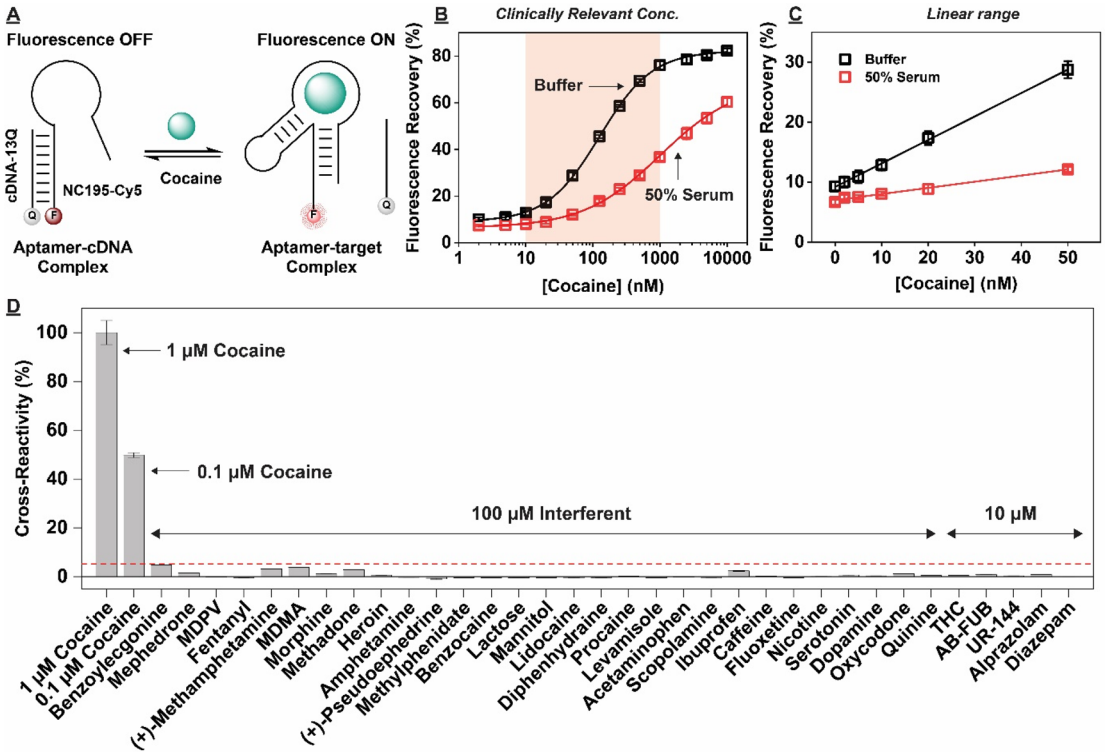

Figure 3. Detection of clinically relevant concentration of cocaine in human blood with an aptamer-based strand-displacement fluorescence sensor. (**A**) Shown here is the working principle of the sensor. (**B**) Calibration curve for cocaine in buffer and 50% human serum. The shaded box indicates the clinically relevant concentration of cocaine in serum. (**C**) Linear range for the sensor (R^2 = 0.99). (**D**) The sensor displays excellent specificity when challenged with 100 μM interferents (certain interferents were tested at 10 μM due to solubility limitations). The dashed red line indicates cross-reactivity of 5% relative to 1 μM cocaine, which, for most of the tested interferents, reflects a 100-fold excess of the interferent over cocaine. Error bars indicate the standard deviations of three independent experiments.

sole, and benzocaine), it is highly likely that this aptamer was eliminated during the counter-SELEX process.

Characterization of Cocaine-Binding Aptamers. The high-stringency selection strategy and metrics used to identify high-affinity aptamer candidates from the high-throughput sequencing data were effective. Using isothermal titration calorimetry (ITC), we determined the binding affinity of our aptamer candidates NC48, NC195, NC423, and NC973 to be 65 ± 9, 22 ± 4, 22 ± 4, and 49 ± 4 nM, respectively (Figure 1E). This represents an order of magnitude improvement over the aptamers we isolated in our low-stringency trial[28] and a more than 200-fold improvement over the widely used MNS4.1 aptamer.[22,37] As a control, we determined the affinities of three aptamers (NC70, NC74.2, NC83) of similar abundance to the selected candidates but of a much lower enrichment fold. Such aptamers had 10-fold poorer affinity (K_D = 300−400 nM) (Figure 1E and SI, Figure S9).

The specificity of the new cocaine-binding aptamers is excellent. To demonstrate this, we used a previously described exonuclease digestion fluorescence assay employing T5 Exonuclease (T5 Exo) and Exonuclease I (Exo I).[32] Here, unbound aptamers are completely digested into mononucleotides by the exonuclease mixture, but ligand-bound aptamers resist digestion to an extent that is dependent on ligand affinity. We monitored the digestion process over time by collecting samples and staining with SYBR Gold to quantify the remaining aptamer concentration. After plotting fluorescence as a function of time, the areas under the curve for a target-free aptamer digestion experiment and aptamer digestion experiments containing a given ligand are used to determine the "resistance value." This is a quantitative measurement of aptamer-ligand binding strength (SI, Figure S10).[32] We assessed the specificity of the candidate aptamers by challenging them with a number of structurally related molecules (benzoylecgonine and scopolamine), drugs of abuse (mephedrone, methylenedioxypyrovalerone, fentanyl, methamphetamine, methylenedioxymethamphetamine, morphine, oxycodone, nicotine, methadone, heroin, amphetamine, methylphenidate, tetrahydrocannabinol, UR-144, AB-FUBINACA, alprazolam, and diazepam), adulterants/cutting agents (benzocaine, lactose, mannitol, lidocaine, diphenhydramine, procaine, levamisole, and pseudoephedrine), commonly used pharmaceuticals (acetaminophen, ibuprofen, quinine, caffeine, and fluoxetine), and endogenous compounds (serotonin and dopamine) (Figure 2A). All four of our aptamers achieved excellent specificity in these tests, with identical response (100% cross-reactivity) to 5 and 100 μM cocaine and minimal cross-reactivity (<10%) to all tested interferents, even at 500 μM. Indeed, even the most cross reactive interferant, the cocaine metabolite benzoylecgonine, exhibited only 50% cross reactivity at a concentration 100-fold higher than that of cocaine (Figure 2B), which is well above its clinically relevant concentration.[23] This level of specificity is far superior to that of a variant of the original MNS4.1 cocaine-binding aptamer (38-GC),[22,38] which responds to cocaine only at a concentration of 100 μM and exhibits ≥50% cross-reactivity to at least 13 different ligands, many of which are quite structurally distinct from cocaine (Figure 2B, 38GC).

Fluorescent Sensor for Detecting Cocaine in Human Serum. Using aptamer NC195, we developed a fluorescent

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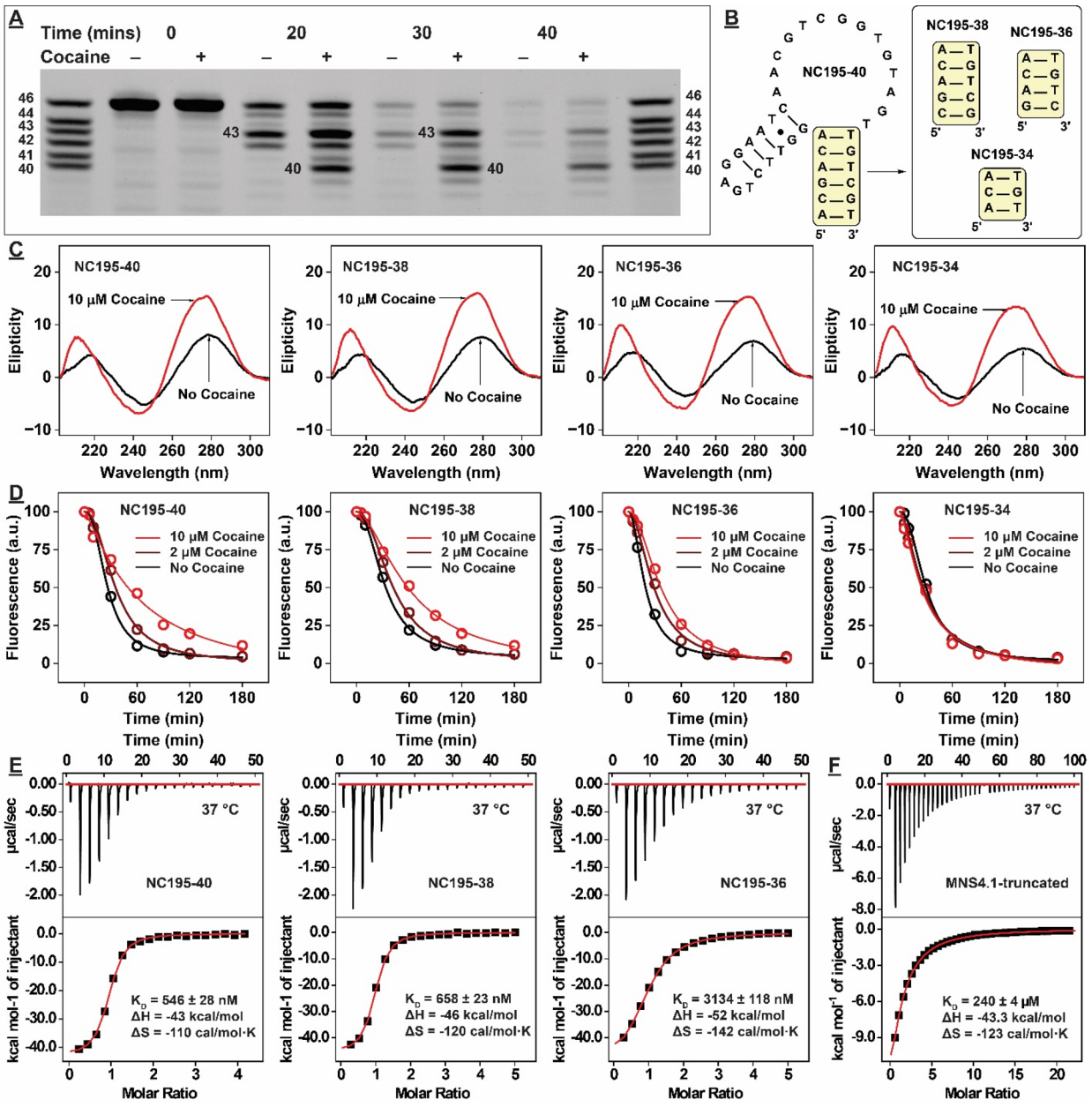

Figure 4. Generation and characterization of a structure-switching cocaine-binding aptamer for adaptation to *in vivo* EAB sensors. (**A**) PAGE analysis of the digestion time-course for NC195 by Exo III and Exo I in the absence and presence of cocaine. The exonucleases generate 43-nt and 40-nt major products. Removing the remaining 5′ overhang from the 43-nt product generates (**B**) the blunt-ended aptamer NC195−40, which we further truncated to generate constructs NC195−38, NC195−36, and NC195−34. (**C**) Circular dichroism spectra of the truncated aptamers. (**D**) Determining the relative cocaine-binding affinity of the truncated aptamers at 37 °C using an Exo I-based assay. (**E**) ITC-based determination of the cocaine-binding affinity of NC195−40, NC195−38, NC195−36, and (**F**) the truncated version of the original cocaine aptamer (MNS4.1-truncated) at 37 °C.

strand-displacement sensor able to quantify cocaine rapidly and conveniently in human blood serum. Here, a fluorophore-labeled aptamer is hybridized with a quencher-labeled complementary DNA (cDNA) sequence; in the absence of the target, this hybridization quenches the fluorophore. In the presence of target, aptamer-target binding releases the cDNA, thereby separating the fluorophore from the quencher and enhancing fluorescence in a target concentration-dependent manner (Figure 3A). Such strand-displacement assays have been shown to support the sensitive and specific detection of various analytes in complex biological samples.[14,15]

We modified NC195 with a 5′ Cy5 fluorophore (NC195-Cy5) and combined it with a cDNA labeled with a 3′ Iowa Black RQ quencher (cDNA13Q). After optimizing the concentration of cDNA13Q, (SI, Figure S11), we challenged aptamer-cDNA complex with cocaine at a concentration range of 0 to 10 μM and assessed the change in Cy5 fluorescence as a function of target concentration. Our sensor achieved a limit of detection (LOD) of 5 nM (the lowest target concentration giving a signal greater than blank + 3σ), a dynamic range of 10−1000 nM (reflecting 10−90% of the maximal signal) that nicely spans the 10−1000 nM clinical range of this drug (Figure 3B,C). This represents an improvement of orders of

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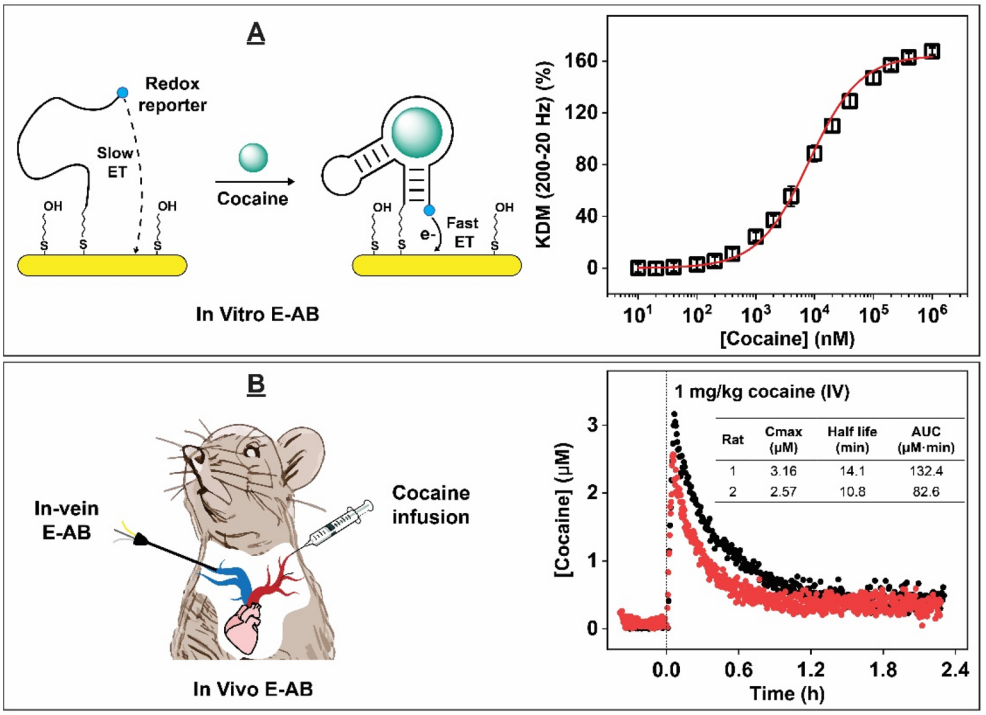

Figure 5. Shown is the real-time, seconds-resolved measurement of cocaine plasma pharmacokinetics *in situ* in the jugular vein of live animals. (**A**) First, we interrogated our *in vitro* EAB sensor in undiluted whole bovine blood at 37 °C using dual-frequency (20 and 200 Hz) square-wave voltammetry to enable kinetic differential measurement (KDM) drift correction. This produced a Langmuir-isotherm binding curve with K_D = 8.0 ± 0.6 μM. (**B**) Upon placement in the jugular veins of two live rats, the sensor exhibited a stable baseline with root-mean-squared noise of 0.11 and 0.04 μM for Rat 1 and Rat 2, respectively. Intravenous dosing of 1 mg kg^{-1} cocaine produced a biphasic pharmacokinetic response that could be measured with excellent precision in individual animals, easily quantifying distinct differences in the pharmacokinetics of the two animals.

magnitude over previous aptamer-based cocaine sensors. For example, the first fluorescence aptamer-beacon cocaine sensor had an LOD of 10 μM, essentially 2000-fold inferior sensitivity.[37] Importantly, our sensor also works well in biological samples, such as serum. To illustrate this, we challenged the sensor with 0−10 μM cocaine spiked into 50% human serum and were able to detect concentrations as low as 10 nM (Figure 3B,C). This again demonstrates a great improvement over previously reported cocaine sensors, such as our fluorescent aptamer-based dye-displacement sensor using 38-GC, which, in just 2.5% serum, achieved only a 900 nM LOD.[38] Finally, assessing the specificity of this sensor against the aforementioned interferents, we observed no cross-reactivities greater than 5% at concentrations 100-fold higher than cocaine (Figure 3D).

Exonuclease-Guided Truncation to Generate Structure-Switching Aptamers. The analytical performance of the strand-displacement sensor motivated us to next adapt NC195 into an EAB sensor format, a versatile platform able to perform continuous, real-time molecular monitoring *in situ* in the living body.[39] This sensor architecture, in which aptamer-ligand binding events generate an electrochemical readout by altering the proximity of a redox reporter relative to a gold electrode, requires an aptamer that undergoes a conformational change upon target binding.[17] To achieve this, we used our exonuclease-guided truncation method, which employs the enzymes Exonuclease III and Exo I to remove nonessential nucleotides from the termini of a ligand-bound aptamer. This digestion process results in a truncated, thermodynamically destabilized product that undergoes binding-induced folding.[40,41] We digested NC195 with a mixture of Exo III and Exo I in the absence or presence of 5 μM cocaine, periodically collecting samples and subjecting them to polyacrylamide gel electrophoresis (PAGE) analysis to identify the length of the truncated products (Figure 4A and SI, Figure S12). In the absence of cocaine, the 46-nt aptamer was completely digested after 40 min. In contrast, products of 40−43-nt persisted in the presence of cocaine, suggesting that the aptamer could be truncated by up to 6 nt while retaining its affinity. We subsequently synthesized the blunt-ended truncated NC195 derivative NC195−40 as well as additional variants from which we removed three, four, or five base pairs, respectively, yielding constructs NC195−38, −36, and −34 (Figure 4B). We opted to use such blunt-ended variants as we have previously observed that aptamers with overhangs often exhibit impaired affinity relative to those without.[16,42] ITC analysis confirmed that the truncated variants retained good binding affinity, with a K_D of 46 ± 5, 83 ± 8, 127 ± 10, and 620 ± 42 nM for NC195−40, −38, −36, and −34, respectively (SI, Figure S13). To confirm if the truncated aptamers have structure-switching functionality, we next used circular dichroism (CD) spectroscopy to monitor the aptamers for binding-induced conformational changes.[43] The spectra of the truncated aptamers all contained positive peaks at 280 and 218 nm and a negative peak at 248 nm. When cocaine was added, the positive peak at 280 nm shifted to 275 nm and grew in magnitude; the negative peak shifted to 240 nm, and the positive peak at 218 nm shifted to 210 nm. These changes indicated that the truncated aptamers undergo a conformational change upon binding to cocaine (Figure 4C).

Our truncated aptamers retain high cocaine-binding affinity under physiological conditions, which is imperative for their

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deployment *in vivo*. We assessed whether our truncated aptamers could bind cocaine in a physiological buffer at 37 °C using an Exo I-based assay. In the absence of cocaine, these aptamers are presumably single-stranded and thus rapidly digested by Exo I into mononucleotides, whereas cocaine-bound aptamers are fully folded and resist digestion. We monitored digestion over time by using SYBR Gold to quantify the remaining intact aptamer molecules. We found that NC195−40, NC195−38, and NC195−36 all resisted digestion in the presence of either 2 or 10 μM cocaine, whereas NC195−34 did not (Figure 4D). We next performed ITC to determine the binding affinity of NC195−40, NC195−38, and NC195−36 at 37 °C in physiological buffer and found that these aptamers bound cocaine with a K_D of 546, 658, and 3134 nM, respectively (Figure 4E). These affinity values contrast sharply with the orders of magnitude poorer affinity ($K_D \approx$ 240 000 nM) of a truncated version of MNS4.1 previously used to fabricate EAB sensors under the same conditions (Figure 4F).[44] Based on the binding profile of NC195−36, we adapted it to the EAB sensor platform. To do so, we attached a methylene blue redox reporter to the 3′ end of the aptamer and modified the other end with a six-carbon thiol group, which was in turn attached to a gold electrode via thiol-gold bonding. We titrated the resulting sensor against cocaine in 37 °C undiluted bovine blood using square-wave voltammetry interrogation at 20 and 200 Hz. We used these two frequencies to compensate for the drift invariably seen under these conditions[18] using kinetic differential measurement (KDM) drift correction,[19] finding that the KDM signal increased monotonically with increasing target concentration (Figure 5A). The useful dynamic range of the sensor spanned from high nanomolar to low micromolar, thus matching the circulating concentrations associated with this drug's psychoactive effects in rats.

Real-Time Cocaine Monitoring in Live Rats. Our EAB sensor supports the continuous, real-time measurement of cocaine in the bloodstream of live animals, providing an unprecedentedly high-resolution view of the drug's pharmacokinetics. To achieve this, we placed EAB sensors into the jugular veins of two live rats (Figure 5B). We observed that the EAB sensors exhibited stable, low-noise baselines (prior to drug challenge, the root-mean-square variance was 0.11 and 0.04 μM for Rat 1 and Rat 2, respectively) and sensitively responded to a behaviorally relevant dose of cocaine administered intravenously (1 mg/kg). The sensor could continuously monitor cocaine over a 2 h with a resolution of ∼14 s, which amounts to 10-fold higher resolution than conventional pharmacokinetic measurements.[6] The pharmacokinetic profiles obtained from our EAB experiments, such as maximum concentration (C_{max}), time to reach this concentration (T_{max}), and time to eliminate 90% of the drug, closely match the values seen in previous studies using conventional approaches to measure cocaine pharmacokinetics.[45] Moreover, the EAB sensor was able to ascertain full pharmacokinetic curves in each individual subject across the lifetime of the cocaine challenge and reveal interindividual differences in pharmacokinetics (see table in Figure 5B).

The *in vivo* EAB sensor is selective for cocaine. To demonstrate this, we performed a series of control experiments with a DNA aptamer that binds to vancomycin. First, we confirmed using isothermal titration calorimetry that the vancomycin aptamer does not have meaningful affinity for cocaine (K_D > 1 mM) (SI, Figure S14). Then, we modified EAB sensors with the 5′ thiolated, 3′ methylene blue-tagged vancomycin aptamer and challenged them with cocaine in buffer at concentrations ranging from 1 nM−320 μM. These sensors did not respond to cocaine at any concentration (SI, Figure S15A). We also implanted this vancomycin EAB sensor in the jugular vein of a rat and administered 1 mg/kg cocaine intravenously and observed no significant change in signal (SI, Figure S15B). These results together indicate that cocaine does not interact nonspecifically with DNA and confirm that our cocaine EAB sensor is detecting cocaine based on selective interaction of the aptamer with its target.

DISCUSSION

Using high-stringency SELEX, we have isolated a set of new aptamers exhibiting exceptional affinity and specificity for the drug of abuse cocaine. These aptamers bind to cocaine with nanomolar affinity and do not respond to a wide range of interfering substances, including other drugs of abuse, endogenous compounds, and commonly used pharmaceutical drugs. We showed that it would be difficult to identify these high-quality aptamers by employing conventional levels of SELEX stringency. However, with unprecedentedly high stringency, wherein SELEX was performed using an ultralow concentration of small-molecule target and reduction of target concentrations by 1000-fold over the course of three rounds of selection, we were able to identify these high-performance aptamers. Using one of these aptamers, NC195, we developed a fluorescence strand-displacement sensor able to detect cocaine in 50% serum with a LOD of 10 nM. This represents a 1000-fold improvement over the performance of the widely used, previously reported cocaine aptamer[37] and is among the lowest ever reported in the literature for the detection of cocaine via a single-step aptasensor. We next introduced structure-switching functionality into our aptamer using an exonuclease-based strategy. Truncated variants of NC195 displayed submicromolar-to-micromolar K_Ds even at 37 °C, a remarkably high affinity for small-molecule-binding, structure-switching aptamers under physiological conditions (∼100-fold better than previously reported constructs).[44] We then utilized the resulting aptamer to develop an EAB sensor, achieving a detection limit 50-fold lower (∼200 nM at 37 °C in blood) than that of an EAB sensor fabricated using the previously reported cocaine aptamer (∼10 μM at room temperature in blood).[46] Implanting this sensor into the jugular veins of live rats, we then demonstrated the seconds-resolved, real-time measurement of the drug in the living body. The resulting data allowed us to clearly identify heterogeneities in peak cocaine concentrations and drug half-lives among individual animals. The limit of detection of our EAB sensor represents the most sensitive continuous analyte monitoring described to date for an *in vivo* electrochemical sensor. It is also noteworthy that this is the first time this drug of abuse (or, indeed, *any* drug of abuse) has been monitored at physiologically relevant concentrations with subminute time-resolution *in vivo*. The aptamers and sensors established here could be of great value for detecting cocaine rapidly and conveniently in clinical settings as well as for studying the neuropharmacology of this drug in live animals.

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■ ASSOCIATED CONTENT

Data Availability Statement

High-throughput DNA sequencing data can be found online at the NIH Sequencing Read Archive by using the name of this paper.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c11350.

Additional methods, sequences of DNA used in this work, selection conditions, summary of ITC data, isolation of cocaine-binding aptamers using library-immobilized SELEX with high selection stringency, polyacrylamide gel electrophoresis analysis of rounds 8−11 of high-stringency SELEX eluents, binding affinity of SELEX pools, proportion of unique sequences in pools, ITC data, enrichment fold plotted against the abundance of sequences for rounds 8−11 of high-stringency selection, scheme for exonuclease digestion fluorescence assay, optimization of quencher complementary DNA for strand-displacement assays, and utilization of a vancomycin EAB sensor to demonstrate the specificity of cocaine detection with EAB sensors (PDF)

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Notes

The authors declare the following competing financial interest(s): KWP owns equity in and consults for companies commercializing in vivo EAB sensors.

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