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Using Exonucleases for Aptamer Characterization, Engineering, and Sensing

Obtin Alkhamis,[†] Juan Canoura,[†] Phuong T. Ly, and Yi Xiao*

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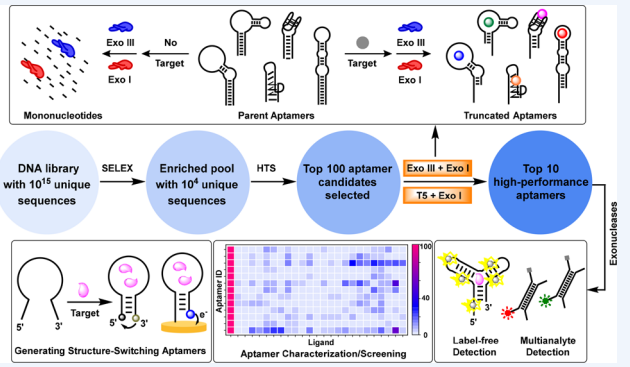
CONSPECTUS: Aptamers are short, single-stranded nucleic acids that have been selected from random libraries to bind specific molecules with high affinity via an *in vitro* method termed systematic evolution of ligands by exponential enrichment (SELEX). They have been generated for diverse targets ranging from metal ions to small molecules to proteins and have demonstrated considerable promise as biorecognition elements in sensors for applications including medical diagnostics, environmental monitoring, food safety, and forensic analysis. While aptamer sensors have made great strides in terms of sensitivity, specificity, turnaround time, and ease of use, several challenges have hindered their broader adoption. These include inadequate sensitivity, bottlenecks in aptamer binding characterization, and the cost and labor associated with aptamer engineering. In this Account, we describe our successes in using nuclease enzymes to address these problems. While working with nucleases to enhance the sensitivity of split aptamer sensors via enzyme-assisted target recycling, we serendipitously discovered that the digestion of DNA aptamers by exonucleases is inhibited when an aptamer is bound to a ligand. This finding served as the foundation for the development of three novel aptamer-related methodologies in our laboratory. First, we used exonucleases to truncate nonessential nucleotides from aptamers to generate structure-switching aptamers in a single step, greatly simplifying the aptamer engineering process. Second, we used exonucleases to develop a label-free aptamer-based detection platform that can utilize aptamers directly obtained from *in vitro* selection to detect analytes with ultralow background and high sensitivity. Through this approach, we were able to detect analytes at nanomolar levels in biological samples, with the capacity for achieving multiplexed detection by using molecular beacons. Finally, we used exonucleases to develop a high throughput means of characterizing aptamer affinity and specificity for a variety of ligands. This approach has enabled more comprehensive analysis of aptamers by greatly increasing the number of aptamer candidates and aptamer-ligand pairs that can be tested in a single experiment. We have also demonstrated the success of this method as a means for identifying new mutant aptamers with augmented binding properties and for quantifying aptamer-target affinity. Our enzymatic technologies can greatly streamline the aptamer characterization and sensor development process, and with the adoption of robotics or liquid handling systems in the future, it should be possible to rapidly identify the most suitable aptamers for a particular application from hundreds to thousands of candidates.



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48, e120.[3] This method is a sensitive high-throughput screening assay utilizing T5 exonuclease for assessing binding between DNA aptamers and small-molecule ligands without any aptamer engineering or labeling requirements.

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INTRODUCTION

Aptamers are short oligonucleotides that bind to specific molecular targets with high affinity.[5,6] Since their initial development roughly 30 years ago, considerable effort has been invested into using aptamers as bioreceptors in sensors.[7,8] Aptamers offer many advantages relative to antibodies, including high stability, low cost of production, ease of chemical modification, minimal batch-to-batch variability, and the ability to modify aptamer affinity and specificity.[9−12] Aptamers are isolated from randomized oligonucleotide libraries through a process termed systematic evolution of ligands by exponential enrichment (SELEX). Since SELEX is performed entirely *in vitro*, aptamers can be isolated to bind targets with specific binding performance metrics in mind (e.g., certain levels of affinity, specificity, or cross-reactivity) or optimized performance under specific conditions (e.g., ionic strength, pH, or temperature).[9] This is in stark contrast to antibodies, which offer no direct control over their binding profiles and can only achieve optimal performance in physiological conditions. Once the sequence of an aptamer has been identified, they can be chemically synthesized reproducibly via solid-phase synthesis techniques and modified with various signaling reporters for different sensing applications. To date, aptamer-based sensors have been applied for purposes including environmental monitoring,[13] food safety,[14] medical diagnostics,[15,16] and therapeutic drug monitoring.[17]

Several technical and logistical challenges have impeded the real-world deployment of aptamer sensors. For example, SELEX typically yields hundreds to thousands of aptamer candidates, from which the aptamer with the appropriate affinity and specificity must be selected. Unfortunately, there are no simple metrics for rapidly identifying such candidates within this vast pool, and thorough screening of many individual aptamers is therefore necessary. Gold-standard methods such as isothermal titration calorimetry (ITC),[18,19] surface plasmon resonance (SPR),[20] and microscale thermophoresis (MST)[21] can provide quantitative binding measurements but have low throughput. As such, aptamers are generally chosen for downstream sensor development based on an inadequate set of binding information. Additionally, because aptamers isolated via SELEX do not have the innate ability to directly transduce target-binding events into measurable signals, engineering steps are generally needed to introduce structure-switching functionality. The resulting conformational changes can then be monitored using various signal reporters, such as fluorescent or electrochemical tags. However, this entails a lengthy trial-and-error process of design, synthesis, and testing to identify aptamer derivatives that offer both high target affinity and the largest target-induced signals.[22,23]

Over the past five years, our group has been utilizing exonuclease enzymes to overcome these hurdles. These works began with the discovery that the digestion of DNA aptamers by exonucleases is inhibited when aptamers bind to their ligand. This finding led to development of an exonuclease-based method to generate truncated structure-switching aptamers that can be directly incorporated into folding-based sensors. We then exploited this exonuclease inhibition phenomenon to develop a novel sensing paradigm that does not rely on structure-switching aptamers, but rather uses fully folded aptamers to enable label-free sensing of multiple targets. We further adapted this exonuclease assay to characterize the binding properties of aptamers at high throughput without any labeling or engineering required. We also utilized this approach to carry out mass screening efforts to rapidly identify mutated aptamers with improved specificity. Finally, we established a strategy for translating the resistance of aptamers to exonuclease digestion into quantitative binding affinity values. In this paper, we provide background for these discoveries, enlighten readers on why and how we pursued these research directions, delineate the utility and benefits of our techniques, and finally conclude with our perspective on the future evolution of such assays.

Early Use of Nucleases in the Aptamer Field

Enzymes have been used to study the structure and function of nucleic acids since 1978, when the first DNA footprinting method was established.[24] In that pioneering work, Galas and Schmitz employed DNase I, an endonuclease that nonspecifically cleaves single- and double-stranded DNA into shorter fragments,[25] to probe interactions between transcription factors and genomic DNA. In 1980, Shalloway and co-workers performed DNA footprinting to study the binding of small molecules to DNA with exonuclease III (Exo III),[26] an exonuclease that selectively digests double-stranded DNA in the 3′-to-5′ direction in a largely sequence-independent manner.[27]

Two decades later, nucleases were first employed to aid in the detection of specific analytes. For instance, in 2005, Lee et al. utilized Exo III in conjunction with SPR imaging to perform ultrasensitive DNA detection.[28] Here, target DNA hybridized with surface-bound complementary DNA probes to enable the digestion of the probes by Exo III, after which the liberated DNA target would hybridize to—and therefore trigger digestion of—another probe. This approach yielded an up to 1,000-fold improvement in detection limits, and was subsequently generalized to the solution phase in 2010 by the Plaxco group, which achieved femtomolar detection limits for DNA.[29] In 2012, the Willner group published an aptamer-based, Exo III-amplified detection strategy.[30] They utilized a specially designed ATP-binding DNA aptamer that contains a 3′ single-stranded overhang and is modified at its 5′ and 3′ termini with a fluorophore and quencher, respectively. In the absence of target, Exo III is unable to digest the aptamer due to the 3′ overhang. When the target is present, the aptamer undergoes a conformational change that yields a 3′ recessed end, allowing Exo III to digest the aptamer, cleaving the quencher from the aptamer and recovering the fluorescence. The authors proposed that the aptamer was digested to release the target for "recycling", wherein the target is captured by another aptamer to repeat the digestion and signaling process.

Our own experience combining aptamers with nucleases began in 2015 while attempting to adapt the target recycling strategy proposed by Willner to gold nanoparticles modified

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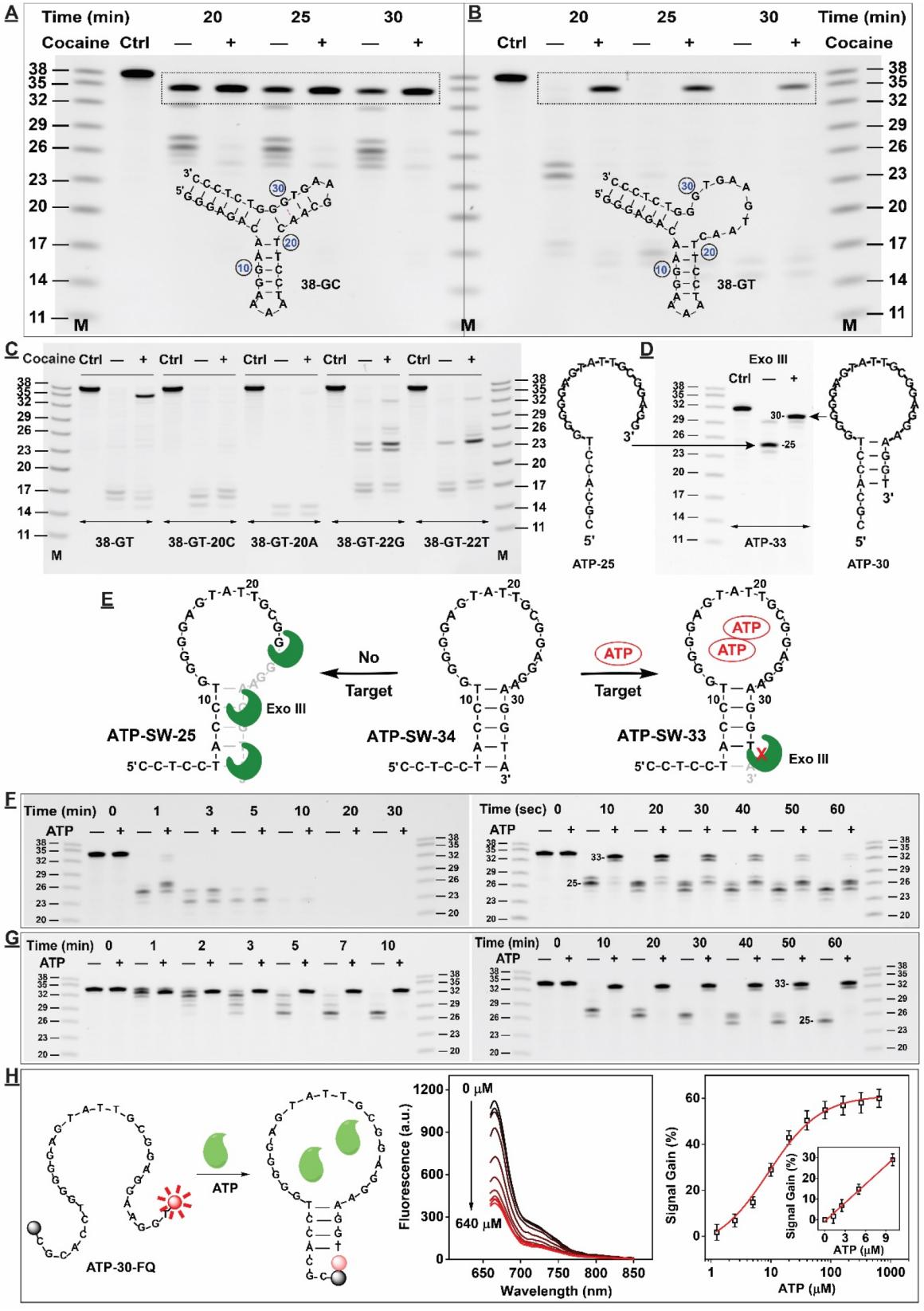

Figure 1. Investigating the exonuclease inhibition phenomenon with DNA aptamers. PAGE analysis of the digestion of cocaine-binding aptamers (A) 38-GC, (B) 38-GT, (C) various 38-GT mutants, and (D) ATP aptamer ATP-33 by Exo III in the absence and presence of their targets. (E) Scheme of Exo III digestion of ATP aptamer ATP-SW-34 in the absence and presence of target, and PAGE analysis of ATP-SW-34 digestion by Exo III in the absence and presence of target at (F) 37 °C with 2 U/μL of Exo III (left: 0−30 min, right: 0−60 s) or (G) 23 °C with 0.05 U/μL of Exo III (left: 0−10 min, right: 0−60 min). (H) Scheme and experimental results for fluorescence detection of ATP using fluorophore-quencher-modified ATP-30. Reprinted with permission from ref 1. Copyright 2018 Oxford University Press.

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with a split cocaine-binding aptamer and Exo III. We covalently attached one fragment of the cocaine aptamer to 13 nm gold nanoparticles via a thiol gold bond, while the other fragment remained free in solution, such that cocaine would induce assembly of the fragments to form aptamer-target complexes on the particle. Exo III would in turn digest the nanoparticle-bound fragment, thereby releasing the target and the free fragment into solution and initiating target recycling. After multiple cycles of digestion, the bare nanoparticles would aggregate, resulting in a red-to-blue color change. A control test in which a complementary DNA was added to the nanoparticle-bound fragment revealed that the surface-tethered DNA could be digested by Exo III and that the nanoparticles could subsequently aggregate to produce a color change. However, when we mixed the nanoparticles with free fragment and cocaine and then added Exo III, we did not observe any color change, indicating that Exo III failed to digest the surface-bound aptamer fragment. This suggested that the interaction between Exo III and aptamers was more complex than originally thought.

A Deeper Examination of Aptamer Digestion by Exonucleases

We subsequently set out to understand the failure of this assay. To determine if the assay worked in solution, we digested the split cocaine aptamer in the absence and presence of cocaine, but obtained inconclusive results. On some occasions, aptamer digestion was accelerated in the presence of cocaine, while other times digestion was unaffected. We hypothesized that the split aptamer was binding to the target too weakly to allow for precise studying of this phenomenon and, therefore, decided to digest the single-stranded parent cocaine aptamer, 38-GC.[31] We assessed the outcome of the digestion reaction using polyacrylamide gel electrophoresis (PAGE), which separates DNA by length. Specifically, we observed how many bands were present in the gel, which indicated the number of products the enzyme generated, and the location of the bands, which is linked to the length of the digestion product. This 38-nt aptamer was digested by Exo III into a 35-nt product regardless of whether cocaine was present or not.[1] In the absence of cocaine, the 35-nt product was further digested into shorter products of 25−29 nt. To our surprise and contrary to what had been reported before, the aptamer seemed to resist further digestion in the presence of cocaine (Figure 1A). To confirm this, we tested aptamer 38-GT, a 38-GC derivative that binds cocaine with similar affinity but contains a G-T mismatch in one of stems and is thus expected to be less thermostable. This experiment revealed even more dramatic differences, wherein the aptamer was nearly completely digested by Exo III in the absence of cocaine but was largely spared in the presence of cocaine (Figure 1B). To confirm that the inhibition of digestion was due to aptamer-target binding, we digested a series of 38-GT mutants with no measurable affinity to cocaine, and observed no significant differences in digestion regardless of the presence or absence of cocaine (Figure 1C). We were curious if the truncated aptamer product of Exo III digestion of 38-GT retained the ability to bind cocaine, and were surprised to determine that the truncated aptamer could indeed bind, albeit with ∼7-fold weaker affinity. These data indicated that when Exo III digests ligand-bound aptamers, digestion is inhibited a few nucleotides prior to the binding domain, and the resulting truncated product remains capable of binding to the target.

We then established the generality of this Exo III inhibition phenomenon with an ATP-binding DNA aptamer,[32] ATP-33. Without ATP, digestion yielded a 25-nt product (ATP-25), which had a K_D of >500 μM for its target. However, in the presence of ATP, the aptamer was digested into a 30-nt product (ATP-30; Figure 1D), which bound ATP with similar affinity to the parent aptamer. In a control experiment, we demonstrated that a mutant with severely impaired target-binding affinity (K_D > 1,000 μM), ATP-33-M, was digested in a similar fashion regardless of the presence or absence of ATP, confirming that aptamer-target binding triggered the inhibition of aptamer digestion.

The fact that aptamer digestion by exonucleases was impeded when the aptamer was bound to a ligand seemed to partially conflict with those of Willner, whom of which observed that aptamer digestion by exonucleases was promoted by aptamer-ligand binding. To clarify these discrepancies, we performed Exo III digestion of the aptamer used by Willner (ATP-SW-34) (Figure 1E, middle) with and without ATP under their and our experimental conditions, and used PAGE to analyze the outcome. Under Willner's conditions (37 °C with 2 U/μL of Exo III),[30] the parent aptamer was almost completely digested within 1 min and no product was detected after 30 min whether ATP was present or absent (Figure 1F, left). We subsequently focused closely on the first minute of digestion, with sampling intervals of 10 s. Interestingly, we observed a clear difference between the digestion of the aptamer with and without ATP within the first 50 s, wherein the aptamer was digested by only 1 nucleotide with the target but was digested down to a 25−27-nt product in the absence of target (Figure 1F, right). Under our experimental conditions (23 °C with 0.05 U/μL of Exo III), we observed significant target-induced inhibition of aptamer digestion beginning as early as 3 min and lasting up to 60 min (Figure 1G). These data together confirmed that inhibition of Exo III by aptamer-target binding occurs to varying extents, regardless of temperature and enzyme concentration.

We hypothesized that Exo III may remove nonessential nucleotides from the termini of aptamers to generate truncated products that, presumably due to their reduced thermostability, have the capability to undergo a large target binding-induced conformational change. To test the functionality of these aptamers, we incorporated the truncation products 35-GT and ATP-30 into an electrochemical aptamer-based sensor for cocaine and a fluorophore-quencher-based ATP sensor, respectively (Figure 1H). These sensors achieved excellent performance, with robust signal gain and low detection limits. This indicated that our exonuclease-based approach could rapidly generate structure-switching aptamers in a single experiment without any foreknowledge of the target binding domain, obviating the need for trial-and-error truncation and testing.

Further Analysis of the Exonuclease Inhibition Phenomenon and Implementation for Detection Purposes

To further explore the generality of this phenomenon, we tested a newer DNA aptamer, DIS-37, that binds the steroid dehydroisoandrosterone-3-sulfate (DIS) with micromolar affinity.[33] This 37-nt aptamer was digested to a shorter 30-nt product without target but enzymatic digestion stalled after removing just 3 or 4 nucleotides when the target was present.[2] Control tests with a nonbinding mutant confirmed that this inhibition was specifically due to aptamer-target binding. We further studied the structure-switching capabilities of these truncated aptamers using another enzyme, exonuclease I (Exo I), which digests single-stranded DNA in the 3′-to-5′

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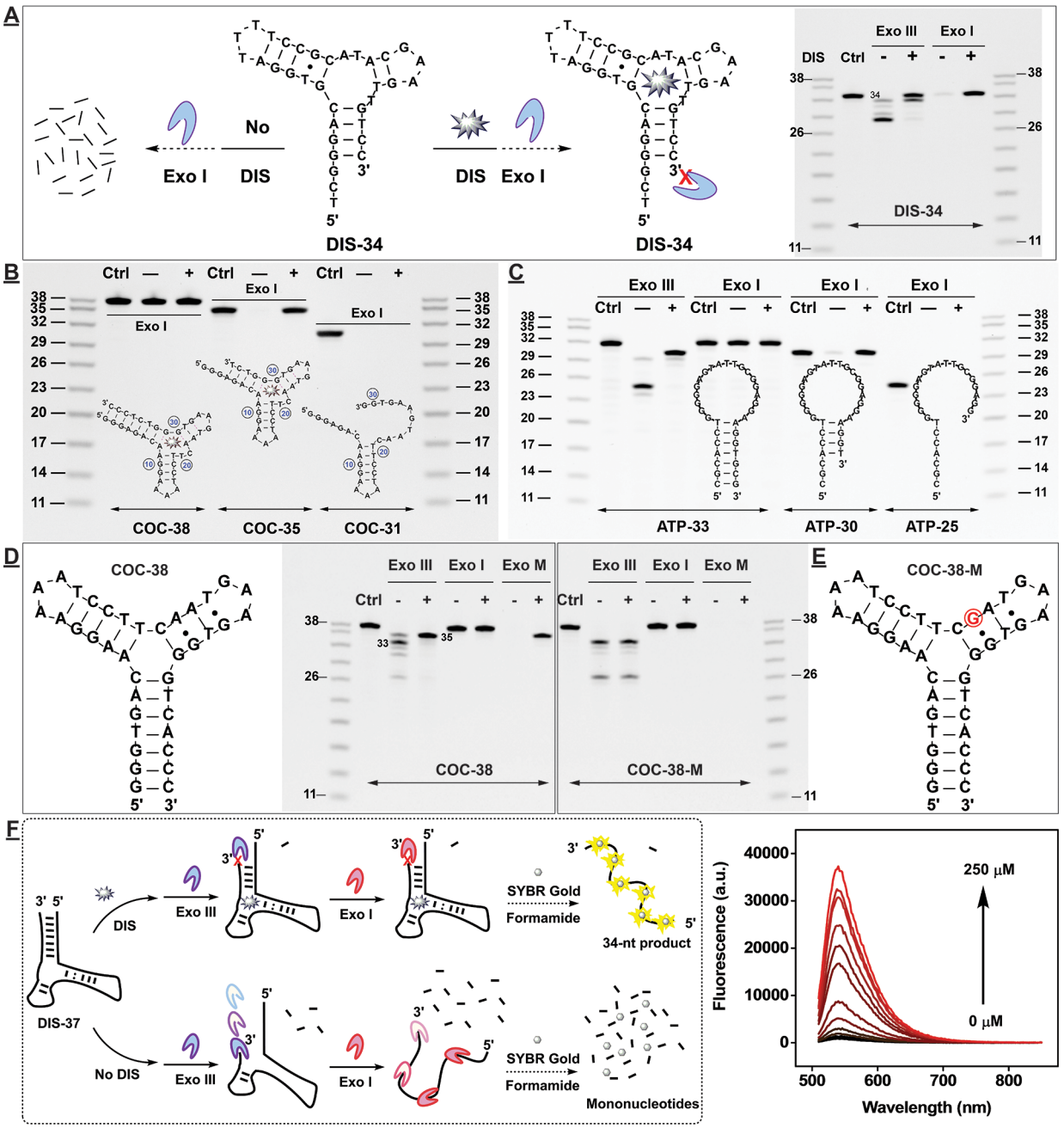

Figure 2. Generality of the aptamer exonuclease inhibition phenomenon and development of the dual-exonuclease fluorescence assay. (A) Scheme of Exo I digestion of truncated aptamers. PAGE analysis of Exo I digestion of (B) COC-38, COC-35, and COC-31 and (C) ATP-33, ATP-30, and ATP-25. PAGE analysis of the digestion of (D) COC-38 and (E) nonbinding mutant COC-38-M using Exo III and Exo I separately and as a mixture (Exo M). (F) Scheme (left) and results (right) of a fluorescent assay based on digestion with Exo III and Exo I using aptamer DIS-37 for the detection of DIS. Reprinted with permission from ref 2. Copyright 2018 American Chemical Society.

direction.[34] Our hypothesis was that the truncated aptamers, which contained partially digested stems that alternate between single- and double-stranded states, would be digested by Exo I in the absence of target since they were unfolded, whereas digestion would be inhibited when the target was added due to switching to a predominately double-stranded folded aptamer-target complex (Figure 2A). This proved true for the truncated aptamer DIS-34 (Figure 2A) as well as the truncated aptamers we generated previously, COC-35 (Figure 2B) and ATP-30 (Figure 2C).

One striking observation from the Exo I digestion experiments was that the truncated aptamers were seemingly being completely digested. We realized that if Exo III and Exo I were employed together in digestion experiments, we could in theory differentiate between the presence and absence of a particular analyte with a high signal-to-noise ratio. To test this, we digested COC-38 with a mixture of Exo III and Exo I without or with cocaine; the aptamer was completely digested without cocaine, but was only digested by three nucleotides in the presence of cocaine, yielding a 35-nt major digestion product (Figure 2D). Notably, we observed no inhibition of digestion when we repeated this experiment with a nonbinding mutant of COC-38 (Figure 2E).

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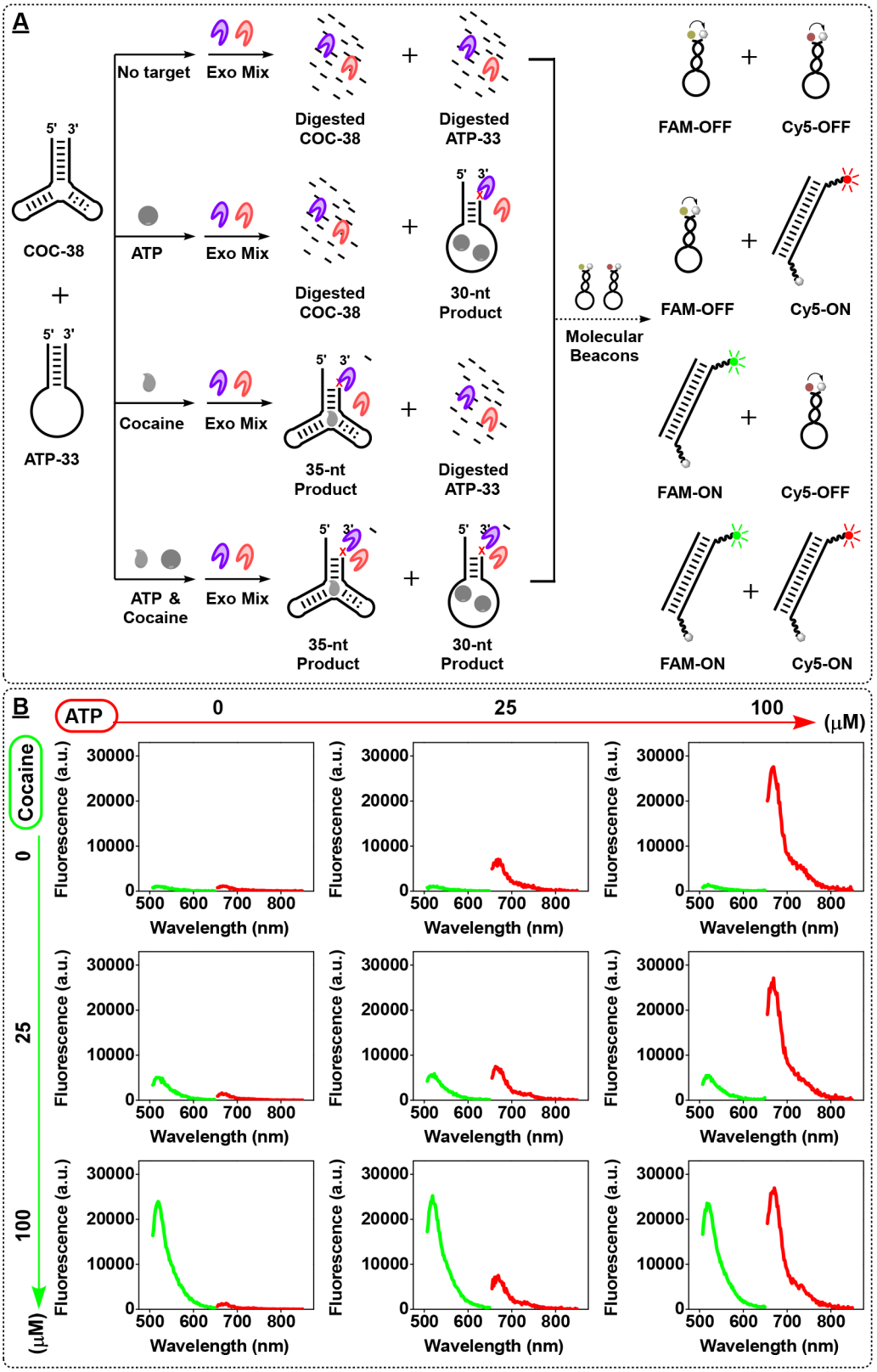

Figure 3. Simultaneous detection of multiple analytes using the dual-exonuclease digestion assay. (A) Schematic depicting possible outcomes of digestion of ATP and cocaine aptamers in the absence or presence of their respective targets and the resulting molecular beacon-based readouts. (B) Fluorescence spectra showing the response of the assay to various samples containing various levels of the two analytes in a mixture. Reprinted with permission from ref 2. Copyright 2018 American Chemical Society.

We saw a clear opportunity to adapt this inhibition effect as an efficient strategy for target detection. To achieve this, we developed a microplate-based assay in which we digested aptamers with the exonuclease mixture, quenched the reaction

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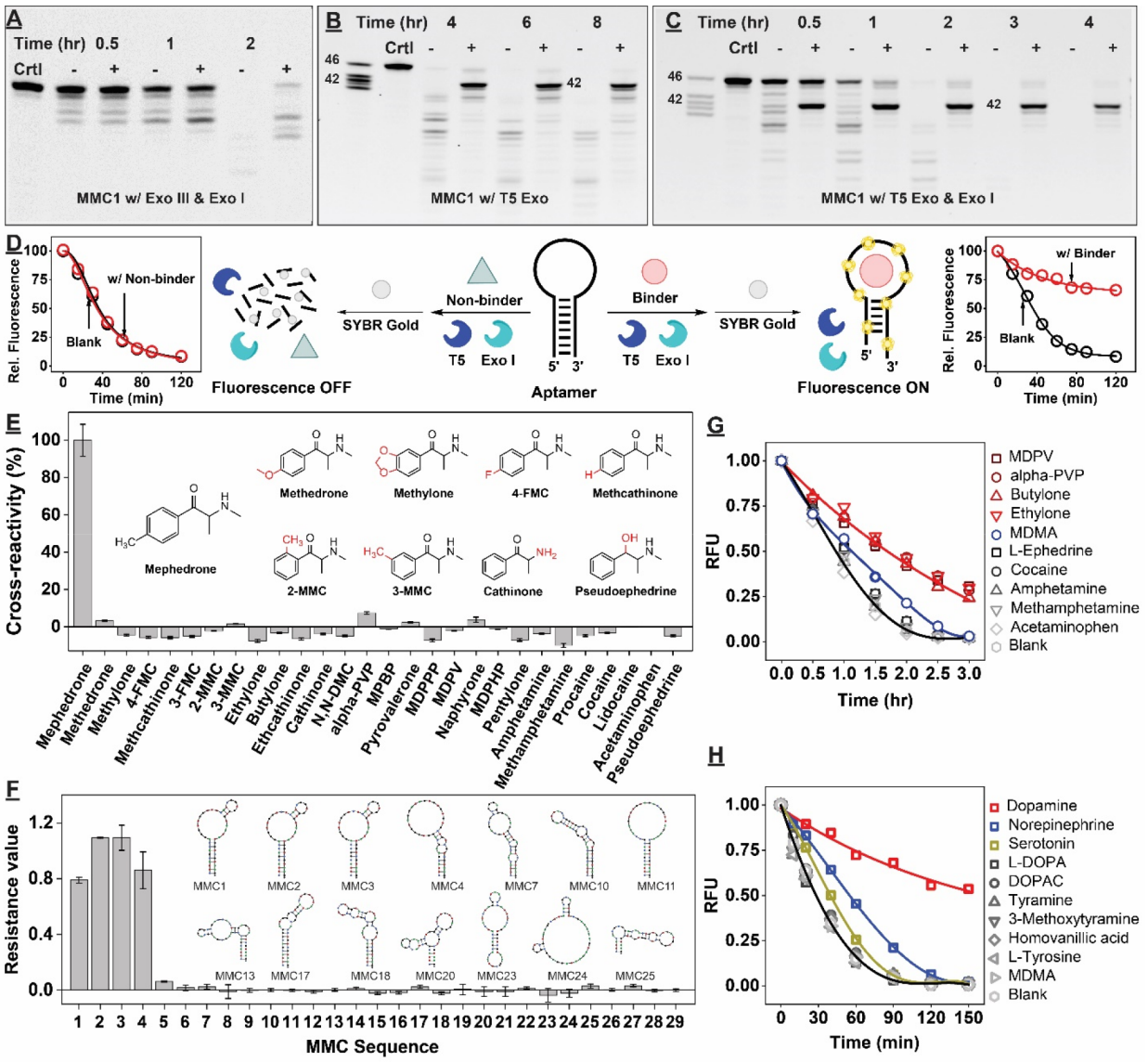

Figure 4. Utilizing T5 Exo to determine the binding properties of aptamers. PAGE analysis of the digestion of mephedrone-binding aptamer MMC1 in the absence and presence of its target with (A) Exo III and Exo I, (B) T5 Exo, and (C) T5 Exo and Exo I. (D) Scheme of the T5 Exo/Exo I aptamer profiling fluorescence assay with simulated results shown at left and right. We used the exonuclease assay to measure (E) cross-reactivity of MMC1 to various synthetic cathinone analogs and (F) capacity of various aptamers to bind mephedrone. Time-course fluorescence plots for the digestion of (G) a synthetic cathinone-binding aptamer and (H) dopamine-binding aptamer in the absence and presence of various ligands. Reprinted with permission from ref 3. Copyright 2020 Oxford University Press.

with EDTA and formamide, and then added SYBR Gold, a dye that selectively binds DNA, to quantify the remaining oligonucleotide products. If the aptamer was completely digested, we would observe minimal fluorescent signal, whereas partially digested, ligand-bound aptamers should bind the dye and thus produce high fluorescence. In an initial demonstration with DIS-37, we indeed observed low fluorescence without target and a large 40-fold increase in signal in the presence of 250 μM DIS. We tested the sensitivity of this assay with varying concentrations of DIS, and could detect DIS at concentrations as low as 500 nM, even in 50% urine samples (Figure 2F). We subsequently demonstrated the generalizability of our approach with cocaine- and ATP-binding aptamers, achieving similar success. These results confirmed that our assay could detect targets with high sensitivity, enabling molecular detection without any need for engineering or labeling of the aptamer itself. We also performed multiplexed detection of ATP and cocaine in a single sample by digesting a mixture of their respective aptamers with Exo III and Exo I. To generate analyte-specific readouts, we used molecular beacons specific to each aptamer's major digestion product, which we labeled with differing fluorophore-quencher pairs (Figure 3A). This allowed us to detect ATP or cocaine at varying concentrations alone or in mixtures without crosstalk (Figure 3B).

Using Exonucleases to Profile Aptamer-Ligand Binding

Around this same time, we were also beginning to isolate new aptamers for small-molecule targets via SELEX to create sensors. One major obstacle we encountered was the large amount of characterization work required to identify the most optimal aptamers for sensor development. By making use of high-throughput sequencing rather than conventional cloning-based sequencing, we were able to identify tens to hundreds of promising aptamer candidates. However, we still had to test the

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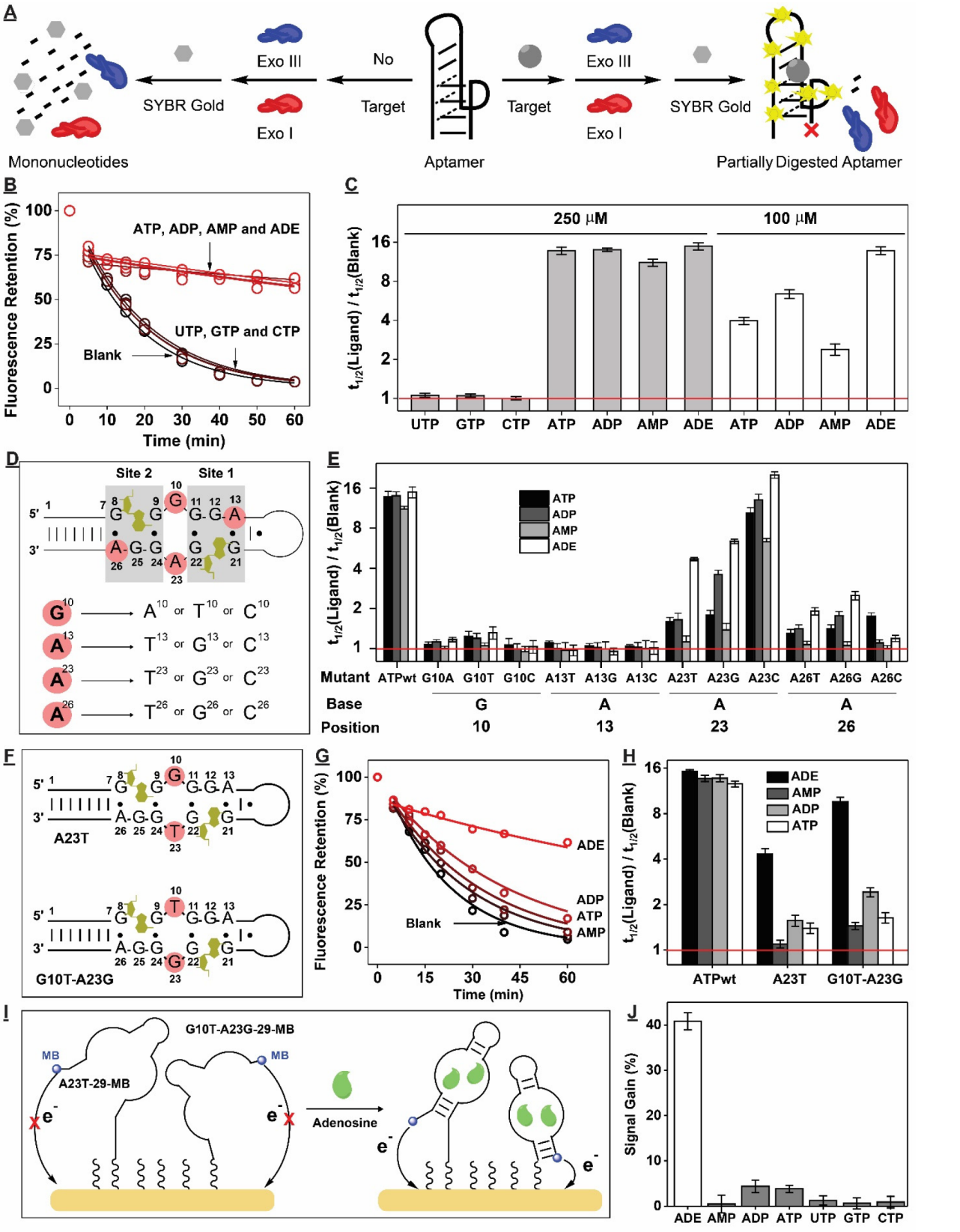

Figure 5. Utilizing the exonuclease assay to screen for mutants of an ATP-binding aptamer with improved specificity. (A) Scheme of the assay. (B) Time-course data for the digestion of ATP-33 in the absence and presence of various nucleoside and nucleotides, with a (C) bar plot depicting corresponding $t_{1/2}$ ratios. (D) Novel ATP aptamer mutants and (E) the binding properties of these mutants according to exonuclease digestion assay data. Adenosine is drawn as a green silhouette in the binding pocket. (F) The highly adenosine-specific aptamers A23T and G10T-A23G, (G) corresponding time-course fluorescence data, and (H) binding profiles. (I) E-AB sensor design for adenosine detection using the aforementioned aptamers and (J) sensor response to adenosine and other analogs. Reprinted with permission from ref 39. Copyright 2021 American Chemical Society.

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binding of these aptamers not only to the target, but to the numerous interferents employed for counter-selection and those relevant to the final sensing application. Gold-standard characterization methodologies like ITC could not achieve the throughput needed for rapid and facile screening of that many aptamer candidates.

One solution we pursued entailed using exonuclease digestion to determine the binding profile of many aptamers in parallel. We knew that aptamer digestion would be inhibited by target binding, but were unsure whether this would hold true for aptamers binding to nontarget molecules and whether the level of inhibition would correlate to aptamer-ligand affinity. To test this, we digested MMC1, a newly identified aptamer for the drug 4-methylmethacathinone (mephedrone),[3] with Exo III and Exo I in the absence or presence of target. To our surprise, we did not observe a significant difference in aptamer digestion with or without target (Figure 4A), even though we confirmed using ITC that it bound mephedrone with K_D = 15 μM. Later on, we determined the cause for this was that these Exo III/Exo I truncated products had weak target affinity, which made them incapable of resisting further digestion by the enzymes. Fortunately, at that time we were testing another exonuclease to study the generality of the aptamer-target binding inhibition phenomenon: T5 exonuclease (T5 Exo), which digests single- and double-stranded DNA in a 5′-to-3′ direction.[35] With T5 Exo, the 46-nt MMC1 aptamer was degraded into shorter oligonucleotides (<40 nt) in the absence of target, but digestion was strongly inhibited in the presence of mephedrone (Figure 4B), yielding a stable 42-nt product that retained affinity for mephedrone (K_D = 7 μM).[3] A control experiment with an MMC1 mutant lacking affinity for mephedrone resulted in no difference in digestion pattern regardless of whether the target was present. This confirmed that, as with Exo III, T5 Exo digestion is inhibited when the aptamer binds to a ligand. We next determined the generality of T5 Exo inhibition with other aptamers with diverse structures, including the G-rich ATP-binding DNA aptamer and our recently isolated three-way-junction-structured MA aptamer,[36] which binds the drug methylenedioxypyrovalerone (MDPV). In all cases, we observed aptamer degradation without target, strong inhibition of digestion in the presence of target resulting in one or two major digestion products, and no difference in digestion pattern with nonfunctional mutant aptamers. These digestion products consistently exhibited the capability to bind the target with similar affinity as the parent aptamer. Furthermore, our experiments testing the ATP aptamer with analogs including adenosine, AMP, ADP, UTP, GTP, and CTP revealed that the level of inhibition seemed to correlate with the strength of aptamer-ligand binding.

We then set out to validate the performance of this binding characterization assay, with two important optimizations. First, since T5 Exo sometimes required >12 h to digest aptamers, we added Exo I to decrease the assay turnaround time. This did not change the digestion pattern relative to that exhibited by T5 Exo alone, but sped the digestion of unbound aptamers (Figure 4C). Second, we adapted the assay into a high-throughput format by utilizing the microplate-based SYBR Gold sensing strategy described above. In addition to digesting aptamers with and without target, we collected samples at multiple time points to monitor the digestion progress. These experiments yielded fluorescence time-course plots that depicted T5 Exo digestion of the aptamer over time (Figure 4D). Using MA as a test bed, we observed that at essentially every time-point, the fluorescence with target was higher than without the target, indicating that the aptamer was resisting digestion. To quantify the degree of enzymatic inhibition, we implemented the "resistance value" metric, which is the ratio of the area under the curve of the time-course plot with target versus without target, minus one. Resistance values of zero or near zero mean that the aptamer is digested at a similar rate regardless of whether or not the target is present, while higher values indicate inhibition associated with aptamer-target binding. We were thus able to assess MA binding to MDPV and more than 20 other ligands (including structural analogues of MDPV and interferents relevant to drug screening) at the same time in a single 2 h experiment, much faster than conventional methods like ITC. Our results showed that MA could bind to interferents such as cocaine and quinine, which we later confirmed using ITC. We also applied this assay to determine the binding profile of MMC1 to mephedrone as well as several interferents and target analogs, confirming that the aptamer had high specificity and no significant cross-reactivity, even to structural isomers, such as 2- and 3-methylmethcathinone (Figure 4E). We also assessed the affinity of nearly 30 newly selected aptamers to mephedrone within just 2 h (Figure 4F). As a final test of generality, we used this assay to accurately profile the binding specificities of a synthetic cathinone-binding aptamer[37] (Figure 4G) and a dopamine aptamer[38] (Figure 4H). These results overall supported the use of T5 Exo as a rapid means for characterizing aptamers without aptamer engineering, labeling, or foreknowledge of the target-binding domain. This profiling method works adequately with aptamers that have nM to μM K_D and inhibition can be observed using ligand concentrations approximately 10-fold greater than the K_D of the aptamer.

Using Exonucleases to Discover New Aptamers with Improved Binding Properties

We realized that one potentially important application of our assay would be the identification of aptamer variants with altered binding profiles. This would allow the discovery of new aptamers without having to perform SELEX. We first validated this approach[39] with several mutants of an ochratoxin A-binding aptamer that had been previously developed and characterized by Xu et al.[40] In the absence of ochratoxin A, the combination of Exo III and Exo I fully digested the aptamers, producing minimal SYBR Gold fluorescence signal. For ochratoxin A-binding aptamers, the presence of this target inhibited exonuclease digestion, generating a strong fluorescent signal (Figure 5A). We assessed these sequences based on the "$t_{1/2}$ ratio", which describes the ratio of the half-life of aptamer digestion in the presence versus the absence of target. We demonstrated that we could sensitively distinguish ochratoxin A-binding sequences based on their high $t_{1/2}$ ratio, whereas weak affinity binders demonstrated a $t_{1/2}$ ratio $\approx$ 1. We also examined the binding profile of the ATP aptamer, and determined (as reported previously)[32] that it can bind ATP, adenosine, AMP, and ADP with similar affinity, but not GTP, CTP, or UTP (Figure 5B−C). We subsequently generated a new panel of 12 point mutants of the ATP aptamer and screened their binding to adenosine, AMP, ADP, and ATP. In a 1 h experiment, we determined that most mutants did not bind any of the ligands, although one exhibited 2−3-fold greater binding to adenosine versus the other analogs (Figure 5D−E). After screening another panel of mutant ATP aptamers, we identified a double mutant with ∼6-fold greater affinity for adenosine relative to the other analogs (Figure 5F−H). Using ITC, we determined that the

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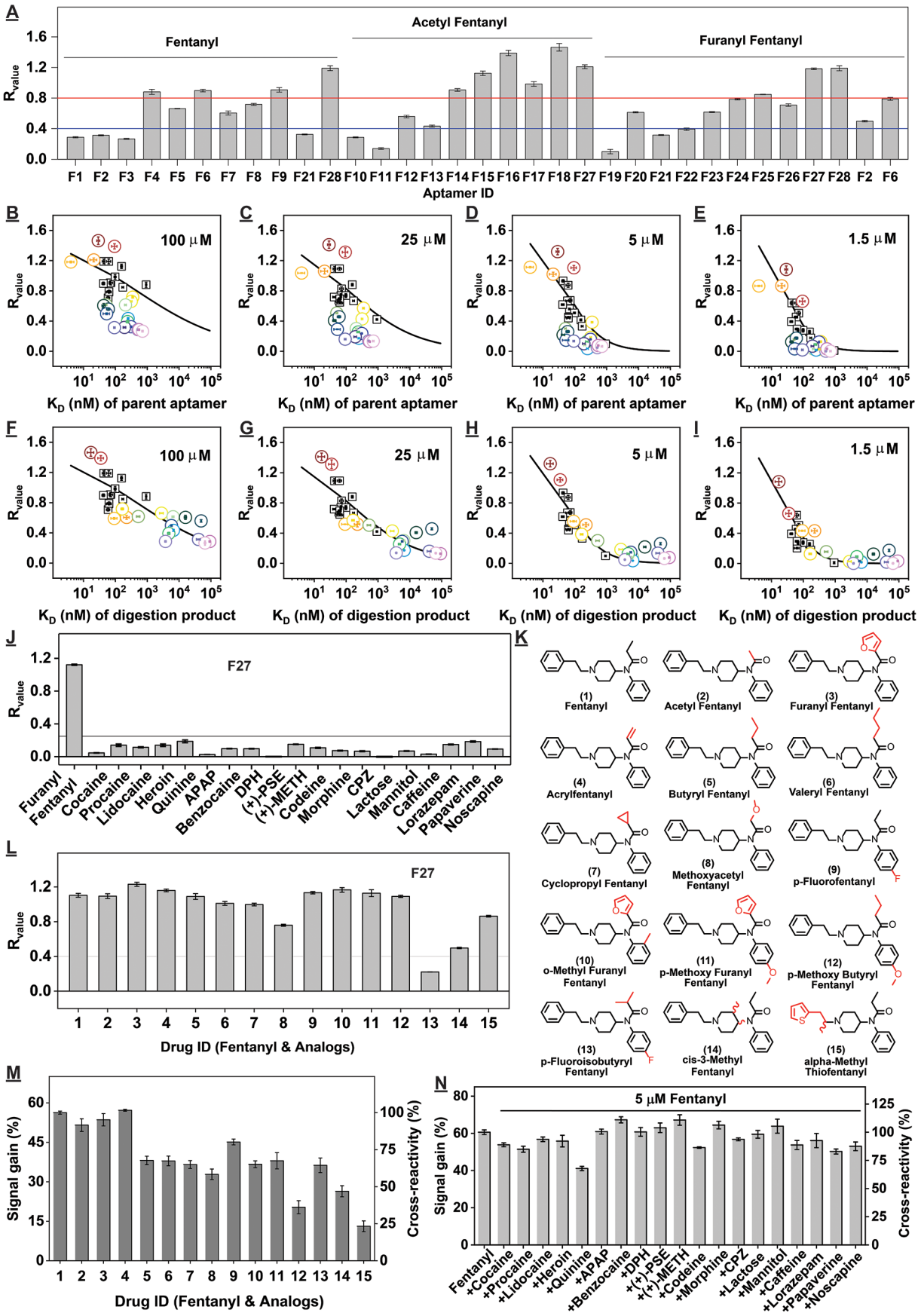

Figure 6. Quantifying the binding strength of aptamers to their targets using exonuclease-based screening. (A) R_{value} of various fentanyl/analog aptamers for their respective target. (B–E) The R_{value} for these aptamers plotted as a function of the K_D of the respective parent aptamer shows poor correlation. (F–I) Plots of R_{value} for each aptamer versus the K_D of its major exonuclease digestion show that R_{value} is an acceptable surrogate for

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Figure 6. continued

measuring the affinity of these truncation products. (J) Specificity of the fentanyl-binding aptamer F27 against diverse interferents. (K) Chemical structures of fentanyl analogs and (L) cross-reactivity of fentanyl-binding aptamer F27 to those analogs. Signal gain and cross-reactivity of an electrochemical sensor based on fentanyl-binding aptamer F13 for (M) 5 μM fentanyl and its analogs or (N) 5 μM fentanyl in binary mixtures comprising 1:40 or 1:100 molar ratios of fentanyl and various interferents. Reprinted with permission from ref 4. Copyright 2022 Oxford University Press.

exonuclease-truncated product of this mutant had a $K_{1/2}$ of 18 μM for adenosine, with at least 10-fold lower affinity for AMP, ADP, and ATP (average $K_{1/2}$ of 180 μM). We confirmed that the truncation products had structure-switching functionality by using circular dichroism spectroscopy. Upon binding of adenosine to the aptamer, we observed a dramatic change in the circular dichroism spectra of the aptamer, indicating that a large conformational change occurred. We then used this aptamer to develop an electrochemical sensor, and confirmed that it had ≤10% cross-reactivity to ADP and ATP relative to adenosine, with nearly no response to AMP, GTP, CTP, and UTP (Figure 5I−J). We could also detect adenosine in 50% serum at concentrations as low as 1 μM, with no interference from other adenosine analogs. Collectively, these results showed that we could use our assay to rapidly carry out mutagenesis screens to find aptamers with improved binding properties for diverse targets.

We also extended this assay to characterize protein-binding aptamers, using DNA aptamers that bind thrombin[41,42] and IgE[43] with low nanomolar affinity. When we digested the thrombin aptamer without target or with a protein not known to bind the aptamer (Factor X), we observed rapid digestion, whereas aptamer digestion was strongly inhibited in the presence of thrombin, indicating tight binding. We likewise demonstrated inhibition of digestion for the IgE aptamer in the presence of human IgE; however no inhibition occurred with human IgG, a ligand for which the aptamer has 1,000-fold poorer affinity.

Quantifying Aptamer Binding Affinity Using Exonucleases

Our work until now had revealed a qualitative relationship between ligand-aptamer binding and resistance to digestion, but we were not yet able to derive a quantitative affinity value (K_D) from this resistance. This required the establishment of a "calibration curve" that correlates K_D with resistance value, ideally based on a variety of aptamers that bind the same target or a class of similar molecules with varying affinity. We therefore performed a series of SELEX experiments to isolate DNA aptamers that bind fentanyl and its analogs acetyl fentanyl and furanyl fentanyl.[4] We identified 28 different aptamer candidates, digested them with 100 μM of their respective target to determine their resistance to Exo III/Exo I digestion, and observed resistance values ranging from 0.2 to 1.4 (Figure 6A). We then measured the affinity of these aptamers with ITC, noting a wide range of K_D values from 4−950 nM. When we plotted these K_D values against the resistance value for each aptamer-ligand pair, we observed no discernible relationship between the two variables (Figure 6B). We also noted two oddities that confirmed the lack of concordance between the two parameters. First, at least 10 aptamers had similar K_D (50−80 nM) but with resistance values ranging from 0.4 to 1.4. Second, several aptamers had the same resistance value of 1.2 but with K_D values spanning 4−100 nM. We repeated the same digestion experiments with lower target concentrations, but still could not correlate the two parameters (Figure 6C−E). We hypothesized that this poor correlation could be attributable to the fact that the digestion products produced during the assay had differing target affinities relative to the parent aptamers. For instance, the cocaine aptamer 35-GT had ∼7-fold poorer affinity for cocaine than 38-GT,[1] while MMC1−42 had ∼3-fold better affinity for its target than parent aptamer MMC1.[3] To test our hypothesis, we identified the digestion products of the fentanyl aptamers using PAGE, and then determined the target affinity of these products with ITC. Although a few digestion products had similar binding affinities as their parent aptamers, most had poorer affinities, with 7−800-fold higher K_D relative to the parent, and in a few rare cases, improved affinity (as much as 2-fold lower K_D). By plotting resistance values against digestion product K_D, we observed a clear trend between these two variables that could be modeled using the Langmuir−Hill equation (Figure 6F−I). The best correlations occurred when we examined a particular range of K_D values at an appropriate target concentration. For example, we could distinguish aptamers with K_D = 10−100 nM most clearly when we used 1.5 μM target for exonuclease digestion, whereas aptamers with K_D = 50−500 nM could be better discriminated using 5 μM target, and 25 μM target was more suitable for aptamers with K_D = 1−100 μM.

Finally, we demonstrated that this assay could determine which aptamers have the highest specificity against 19 diverse interferents. After testing 560 aptamer−ligand pairs, we identified aptamers that solely recognized fentanyl or its analogs but not other illicit drugs, adulterants, and cutting agents (Figure 6J). We then determined the cross-reactivity of aptamers for 14 diverse analogs of fentanyl (Figure 6K) using the exonuclease digestion assay and identified that F27 has a broad cross-reactivity to analogs (Figure 6L). We utilized the best of these aptamers to detect fentanyl and its analogs using electrochemical aptamer-based sensors, achieving a limit of detection of 15 nM in 50% saliva, and could even detect 1% fentanyl (by molarity) in binary mixtures containing various interferents (Figure 6M−N). These results showcase how the exonuclease digestion assay can streamline aptamer-based sensor development, yielding structure-switching aptamers with defined K_D values and specificity profiles that can be readily incorporated into various sensor platforms.

■ CONCLUSION AND OUTLOOK

Here, we have recounted the discovery of the exonuclease inhibition phenomenon and how we have applied it to generate structure-switching aptamers, perform multianalyte sensing with aptamers, and characterize the binding properties of aptamers both qualitatively and quantitatively. Given the high-throughput nature of this assay, numerous samples can be processed at once, and we can typically estimate K_Ds for 12 different aptamers in one experiment within 1−2 h. We have assessed up to 48 aptamer-ligand pairs in a single experiment, with throughput limited primarily by instrumentation (*e.g.*, capacity of multichannel pipettes or microplates). Despite this, our assay has much higher throughput than gold-standard methodologies like ITC, MST, and SPR. Notably, the exonuclease technologies

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described here are beginning to be used by researchers to assess aptamer affinity[44] and detect targets with aptamers.[45,46]

We envision several future directions for the further improvement of this assay. First, throughput could be further increased with robotic liquid-handling systems. This could enable mass screening of newly isolated aptamer candidates to identify those with optimal binding properties for a given application, or expedite mass screens of hundreds or thousands of aptamer mutants to find sequences with augmented binding performance. We are also working on enhancing assay efficiency. Our assay currently requires sample collection at various time-points for each aptamer−ligand pair to quench the enzymatic reaction and stain the aptamers with SYBR Gold, which is labor- and resource-intensive, increases assay error, and potentially reduces throughput. We are therefore designing new ways to monitor aptamer digestion in real time, thereby reducing the assay to a single mix-and-observe step. Finally, we are pushing toward using the assay to study aptamer binding kinetics. Unlike methods for K_D determination, there are relatively few methods available for studying binding kinetics, especially for the interaction between aptamers and small molecules. Our preliminary data indicate that aptamer digestion rate is impacted by the kinetics as well as the thermodynamics of aptamer-target binding. Deriving this kinetic information from the exonuclease digestion assay will require further modifications of experimental protocols and new modes of data analysis; the resulting data will be of great use for aptamer characterization.

AUTHOR INFORMATION

Corresponding Author

Yi Xiao − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0001-7278-9811; Email: yxiao34@ncsu.edu

Authors

Obtin Alkhamis − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Juan Canoura − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States*

Phuong T. Ly − *Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0003-2788-4443

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.accounts.3c00113

Author Contributions

†These authors contributed equally to this work. CRediT: **Obtin Alkhamis** data curation (lead), formal analysis (lead), investigation (lead), methodology (lead), writing-original draft (lead), writing-review & editing (lead); **Juan Canoura** data curation (lead), formal analysis (lead), investigation (lead), methodology (lead), software (equal), writing-original draft (equal); **Phuong Ly** software (lead), validation (lead), writing-original draft (supporting); **Yi Xiao** conceptualization (lead), funding acquisition (lead), project administration (lead), supervision (lead), writing-review & editing (lead).

Notes

The authors declare no competing financial interest.

Biographies

Obtin Alkhamis received his bachelor's degree in chemistry from Florida International University in 2017. He is currently a Ph.D. candidate working in the laboratory of Dr. Yi Xiao in the Department of Chemistry at North Carolina State University. His research is focused on the development of new approaches to generate high-affinity DNA aptamers and utilize those aptamers to develop sensors that can detect analytes for on-site or point-of-care applications.

Juan Canoura received his bachelor's degree and Ph.D. in chemistry from Florida International University. During his Ph.D., he investigated the capability of exonuclease mixtures to distinguish ligand-bound from unbound aptamers. He has applied this phenomenon towards the screening of aptamer binding properties and engineering signal-reporting aptamers for subsequent sensor development.

Phuong T. Ly received her B.S. in Chemistry from Ho Chi Minh City (HCMC) University of Science in 2021 studying natural compounds from medicinal plants. She is currently a graduate student working in Dr. Yi Xiao's research lab in the Department of Chemistry at NC State University, and her main direction is focusing on isolating aptamers with high affinity and specificity via SELEX and utilizing them as bioreceptors in various bioassays.

Yi Xiao received her B.S. in the department of Chemistry from Northwest University in China and her Ph.D. in Analytical Chemistry from Nanjing University in China. She is an Associate Professor of Chemistry at North Carolina State University. Her current research interests are in the development of innovative methods for the rapid isolation, characterization, and engineering of high-quality DNA-based affinity reagents—particularly aptamers—for sensing and therapeutic applications.

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