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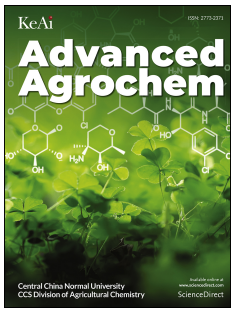

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Competitive Aptamer Switch for Modulating Ligand Binding Affinity

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KEYWORDS

aptamer-complement duplex aptamer switches competitive hybridization molecular sensing

Abstract

Aptamers are short, single-stranded DNA or RNA molecules that selectively bind to a target molecule. Aptamer-complement duplex (ACD) is often used to design molecular switches capable of producing a detectable signal or triggering a structural change upon aptamer binding to a target. However, such aptamer switch generally faces an increased dissociation constant (K_d) due to the energy barrier of the complementary duplex. We reported a competitive hybridization mechanism to modulate the binding affinity of an ACD to a target adenosine. Using the computation-guided design, we calculated the aptamer folding energy for the duplex length from 11-nt to 15-nt, and experimentally measured increased apparent K_d values resulted from these extended duplexes. Using a set of strands to compete with the ACD hybridization, it reduced the aptamer folding energy to facilitate aptamer switches with decreased apparent K_d values ranging from over 400 μM without a competing strand to ~ 30 μM with a competing strand. This competitive aptamer switch was also found sensitive to single-nucleotide mutations of a competing strand. Our work provides an approach to modulate the binding affinity and the sensitivity of aptamer-complement duplexes, which could be useful in the nucleic acids-based sensing and nanomedicine.

Introduction

Aptamers are short, single-stranded oligonucleotides that fold into a secondary structure for selectively binding to a molecular target. In more than 3 billion years of life evolution, biology uses ligand-binding RNA molecules to regulate the gene transcription and translation in cellular metabolism, e.g. riboswitch.[1] In early 1990s, the in vitro selection of RNA molecules was demonstrated for binding to specific small-molecule ligands.[2, 3] SELEX (Systematic evolution of ligands by exponential enrichment) is the most commonly used method to select DNA or RNA sequences for binding to a target with K_d (dissociate constant) ranged from pM to μM.[3] Aptamers have been found quite useful in multiple applications of molecular sensing,[4, 5] imaging,[4] nanomedicine[6, 7] and biocatalysis.[8, 9] Especially, aptamers find utility in agricultural applications for the rapid detection of chemical contaminations in farming lands and agricultural products, including antibiotics, pesticides, toxins, and other hazardous chemicals such as heavy metals.[10] Aptamers are also useful in sensing the presence of bacterial pathogen in various food products of vegetables, fruits and meats.[11]

Structurally switching aptamers are particularly useful for these applications. Such aptamers are able to alter the structure upon binding to a target molecule, releasing signals for detection or triggering follow-up functions.[6, 12] Aptamer-complement duplex (ACD) is the most commonly adopted strategy for designing structurally switching aptamers, in which an aptamer is partially hybridized to a complementary strand. When ACD binds to a target molecule, the duplex is disrupted to release the complementary strand. By incorporating fluorescent or plasmonic probes,[5, 13] the disruption of the duplex can produce signal for detection.[14, 15] ACD was also demonstrated to detect various targets with triggering an enzymatic glucose production.[16] Moreover, ACD provided a mechanism to develop theragnostic nanodevices for smart drug delivery. For example,

Douglas et al. demonstrated a DNA nanorobot with ACD locks capable of specially recognizing leukemia cancer cell and releasing payloads to kill it.[6] ACD-locked DNA origami was also used as vaccine for cancer immunotherapy.[17] In addition, ACDs are reported to modulate enzyme-substrate binding for developing biomimetic catalytic complexes.[8, 18]

Despite broad applications of ACDs, such structures face a problem of decreased binding affinity due to the energy barrier of the complementary duplex. The apparent K_d of an ACD is generally 1-2 magnitudes higher than a native aptamer,[12, 16] and some stable ACDs even prevented aptamers from binding to a target.[8] Thus, the ability to modulate the binding affinity of ACDs to target molecules will promote their usage and applications. A recent study used toehold-mediate activation to trigger the aptamer switch for improving the thermal robustness and programmability of aptamers.[19] Here, we used a competitive hybridization to modulate ACD-target binding, in which a competing (CP) strand facilitates the structural switch to release a native aptamer for binding to a target molecule. Using the computation-guided design, we evaluated a set of CP strands for impacting the sensitivity of aptamer-complement switches and recognizing single-nucleotide mutations.

Materials and Methods

Materials: DEAE Sepharose resin, Sigma prep column, dimethylformamide (DMF), and adenosine were purchased from Sigma-Aldrich (St. Louis, MI, USA). Amicon Ultra-4mL filter was obtained from VWR international (Radnor, PA, USA). Magnesium chloride ($MgCl_2$), 10 × tris buffered saline (TBS) buffer and DNA grade water were ordered from Fisher Scientific (Pittsburgh, PA, USA). All oligonucleotides were produced by Integrated DNA Technologies (Coralville, IA, USA), and their sequences were listed in the Supporting Information **Table S1-3**.

NHS-Cy3 and NHS-Cy5 were obtained from Lumiprobe (Hunt Valley, USA) and Alexa488 was purchased from Fisher.

Reagent Preparation: DNA oligonucleotides were dissolved in DNA grade water, and concentrations of DNA stock solutions were quantified by 260 nm absorbance using NanoDrop 2000 (Thermo Scientific, Waltham, USA). Analytical grade or molecular biology reagents and distilled (DI) water were used in all experiments unless otherwise specified. Stock solutions of organic dyes were prepared in water-free DMSO.

DNA labelling with organic dyes: 200 μL of 100 μM amine-modified DNA solution was prepared in pH 8.5, 100 mM HEPES buffer. 20 × excess NHS conjugation dye (e.g. Cy3, Cy5 or Alexa488) was added into DNA solution to allow an incubation on a shaker (500 rpm) for 1 hour at room temperature in the dark. DEAE resin was used to remove excess, unconjugated dyes from DNA solutions as previously described.[20, 21] Collected dye-conjugated DNA solution was washed into DNA grade water by centrifugation with a 3 kDa Amicon-Ultra filter.

Preparation of aptamer-complement duplexes and binding assays: Aptamer and complementary strands were prepared in 1 × TBS (pH 7.5), 2 mM $MgCl_2$ buffer and were annealed in a thermocycler from 90 ^{o}C to 25 ^{o}C (**Table S4**).[21] In the titration of Mg^{2+} concentrations, 1 × TBS, 2 mM $MgCl_2$ gave the best result for ACD binding to adenosine (supporting information **Figure S1**). For binding assays, a set of adenosine solutions were first added into 100 nM ACDs with the incubation in the dark for ~ 30 min. Then, fluorescence signal was detected by a Biotek Cytation 3 plate reader with the Cy3 channel of 530 nm excitation and 570 nm emission. For the competitive hybridization, 100 nM CP strand was first added into ACD solution, followed by the addition of adenosine. All reaction curves and fittings were analyzed by GraphPad Prism software.

In silico NUPACK simulation: NUPACK was used to simulate DNA hybridization and energy for aptamer-complement duplexes. For most simulations, the temperature was set at 25 ^{o}C and Na^{+} was kept at 0.137 M. Mg^{2+} were set at different concentrations from 0 to 0.016 M based on specific experiments. The DNA simulation parameters were described by "SantaLucia, 1998" in NUPACK.

Results and Discussion

As shown in **Figure 1A**, a native aptamer (APT) generally folds into a secondary structure with one or more loops under appropriate ion and pH conditions. The structure recognizes a ligand and binds to it (e.g. adenosine). For an ACD, an aptamer is partially hybridized to a complementary strand to form a duplex. This duplex can still bind to a target molecule, however, will undergo a disruption of the complementary duplex to release a folded aptamer binding with the target. For sensing, the duplex is usually labelled with a pair of fluorophores with FRET or quenching, and a change of fluorescence signal is detected when the duplex is disrupted. The increased hybridization energy of an ACD makes it more difficult to release a folded aptamer, thus, ACD binds to a ligand at a much higher K_d than the native aptamer. For example, a selected aptamer binds to adenosine at $K_d \sim 6$ μM,[8, 9, 22] while the ACD binds to adenosine at K_d of several hundreds of μM. In **Figure 1B**, a CP strand is added that competes with the aptamer for hybridizing with the complement strand. This competitive hybridization facilitates the structural switch of an ACD to release an actively folded aptamer for binding with a ligand.

In **Figure 2A**, we used NUPACK to calculate the folding energy of aptamers (ΔG (APT Fold)) for different aptamer-complement duplexes, based on simplified equation 1 -3 below:

$$\Delta G(APT\ Fold)^1 = \Delta G(APT) \quad (1)$$

$$\Delta G(APT\ Fold)^2 = \Delta G(APT) - \Delta G(ACD) \quad (2)$$

$$\Delta G(APT\ Fold)^3 = \Delta G(APT) + \Delta G(CP - CL) - \Delta G(ACD) \quad (3)$$

For a native aptamer, ΔG (APT Fold)[1] is estimated as the folding energy of an aptamer. In equation 2, for an ACD, ΔG (APT Fold)[2] is estimated by the folding energy of an aptamer (ΔG(APT)) subtracting the hybridization energy of the complement duplex (ΔG(ACD). In equation 3, upon the addition of a CP strand, the folding energy (ΔG (APT Fold)[3]) is revised to the sum of a native aptamer folding ΔG(APT) and the hybridization energy of a CP-CL duplex (ΔG(CP-CL)) with the subtraction of the hybridization energy of the ACD (ΔG(ACD)). A native aptamer spontaneously folds into a secondary structure with ΔG~ -2.6 kcal/mole, while a 11-bp ACD hardly releases a folded aptamer with a positive ΔG ~ 16.7 kcal/mole. Increasement of the duplex length will result in more positive ΔG for aptamer folding, e.g., ~ 17.1 kcal/mole for 12-bp duplex, ~ 19.3 kcal/mole for 13-bp duplex and ~ 22.5 kcal/mole for 15-bp duplex. The increased positive ΔG folding makes the structure more difficult to release an aptamer upon binding with an adenosine. This was validated experimentally, where a native aptamer bound to an adenosine at a K_d ~ 6 μM (supporting information **Figure S2**), and a 11-bp ACD bound to an adenosine at a much higher K_d ~ 450 μM (**Figure 2B**). Further increase of the duplex length resulted in even higher K_d of ~ 635 μM for 12-bp duplex and ~ 2100 μM for 13-bp duplex. 15-bp ACD was unable to bind with an adenosine.

To reduce K_d of an ACD for binding with an adenosine, we tested the addition of CP strands to facilitate the structural switch. In **Figure 3A**, the aptamer folding energy was decreased from 16.7 kcal/mole for a 11-bp ACD, to 4.1 kcal/mole by adding a 8-nt CP, 2.1 kcal/mole by adding a 9-nt CP, 0.1 kcal/mole by adding a 10-nt CP and -1.1 kcal/mole by adding a 11-nt CP. The corresponded K_d of a 11-bp ACD binding to adenosine was reduced from ~ 450 μM for a 11-bp

ACD, to ~ 150 μM by adding a 8-nt CP, ~ 110 μM by adding a 9-nt CP, ~ 102 μM by adding a 10-nt CP and ~ 26 μM by adding a 11-nt CP (**Figure 3B**). Similarly, the aptamer folding energy for 12-bp ACD was reduced from 17.1 kcal/mole to 4.7 kcal/mole by adding a 8-nt CP, 2.7 kcal/mole by adding a 9-nt CP, 0.8 kcal/mole by adding a 10-nt CP, - 0.5 kcal/mole by adding a 11-nt CP and – 1.4 kcal/mole by adding a 12-nt CP. The corresponded K_d was decreased from ~ 620 μM for a 12-bp ACD duplex to ~ 440 μM by adding 8-nt CP, ~ 230 μM by adding 9-nt CP, ~ 140 μM by adding 10-nt CP, ~ 87 μM by adding 11-nt CP and ~ 50 μM by adding 12-nt CP (**Figure 3C and D**). In **Figure 3E and F**, the aptamer folding energy of a 13-bp ACD was also reduced by adding CPs and the K_d was decreased from 2200 μM without a CP strand to 45 μM with the addition of a 13-nt CP. For the sensitivity of detection, we calculated S/N (signal to noise ratio) for detecting adenosine by 11-bp ACD and 11-bp ACD + CP. For a noncompetitive 11-bp ACD, the S/N was ~ 2.5 for detecting 15. 6 μM adenosine, and ~ 7.3 for detecting 31.2 μM adenosine. For a competitive 11-bp ACD + CP, the S/N was ~ 9.3 for detecting 15. 6 μM adenosine, and ~ 16.3 for detecting 31.2 μM adenosine. The above results demonstrated the improvement of S/N and detection limit for detecting low concentrations of adenosine.

In supporting information **Figure S3**, we showed that the K_d of an ACD binding to a NAD^+ was reduced from ~ 1800 μM to ~ 400 μM by adding a CP strand. The above experiments demonstrated that the binding of ACD to a target molecule could be well modulated by using competitive hybridization, and ACDs were easier to sense and bind to target ligands by adding CP strands. To be noted, the addition of competing strands increases the background fluorescence of ADC, and it needs to balance between the decreased K_d of ligand binding and the increased background fluorescence. The ratio of CP to ACD also impacted the equilibrium of aptamer switches. The addition of more competing strands (or a high ratio of CP to ACD) is expected to facilitate the

release of an aptamer from ACD, however, the high ratio of CP to ACD also destabilizes the ACD structure (supporting information **Figure S4**). CP strands also slightly increased the speed to reach reach the equilibrium of aptamer switch (supporting information **Figure S5**).

Next**,** we tested how single-nucleotide mutations (SNMs) of a CP strand would impact an ACD binding to an adenosine. In **Figure 4A**, using a 11-bp ACD, SNMs of all positions at a CP strand were calculated for affecting the aptamer folding energy. SNMs increased ΔG of the aptamer folding, varied from -1 kcal/mole for a wild type (WT) CP to > 4 kcal/mole for a 5C/A mutation. In **Figure 4B**, apparent K_d values were experimentally measured for CP strands containing these SNMs, that K_d was increased from ~ 25 μM for a WT CP to > 200 μM for 3T/G and 5 C/A mutations. Overall, the trend of ΔG variation for the aptamer folding corresponded to the increased apparent K_d measured for SNMs of a CP strand. More data was shown in supporting information Figure S6.

Conclusion

In summary, we investigated how the folding energy of an aptamer was affected by the duplex length of an ACD, as well as the K_d values of ACDs binding to a target adenosine. Using a CP strand, it facilitated the folding switch of an aptamer and decreased the apparent K_d of an ACD binding to a target molecule. Further, the SNMs of a CP strand increased the aptamer folding energy in an ACD, resulting in increased K_d values of the aptamer switch for binding to the target molecule. This competitive hybridization provides new insights into the aptamer switch, could be useful to improve aptamer-based applications in chemical sensing, agricultural and environmental safety, and therapeutics.

Figures

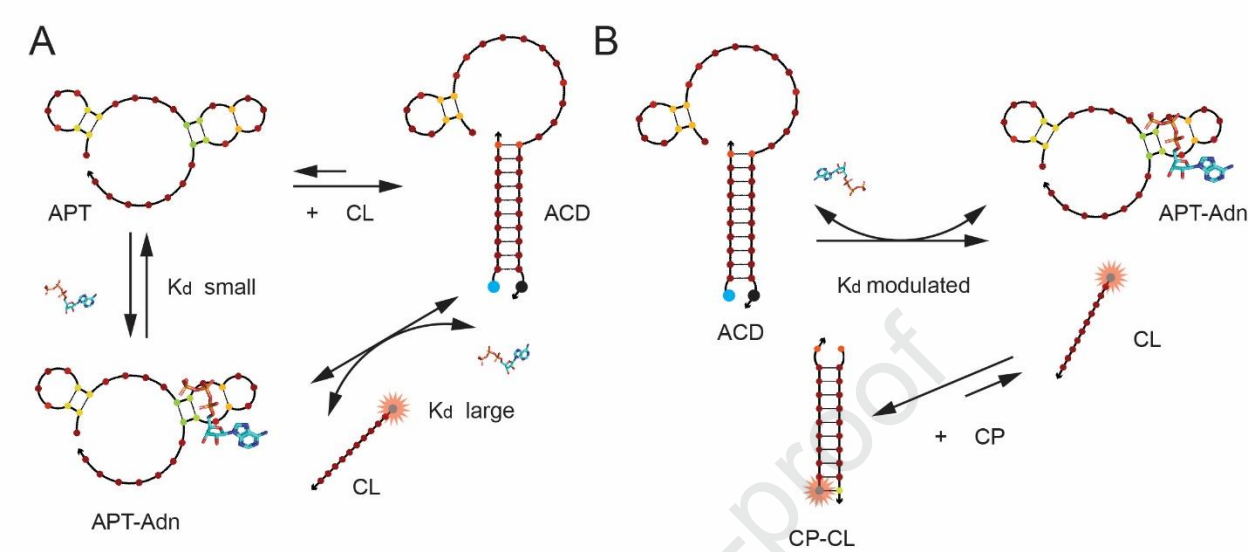

Figure 1. Mechanistic concept of competitive aptamer switch. (A) Aptamer (APT) folds into a conformation binding to a ligand (adenosine, Adn) with a small K_d. An aptamer-complement duplex (ACD) is formed by hybridizing an APT with a complementary strand (CL), which duplex is disrupted by adding a high concentration of ligand to trigger the structural switch. However, such an ACD generally shows an increased K_d for binding to a ligand. (B) The addition of a competitive strand (CP) to efficiently remove free CL to form a stable CP-CL duplex, which promotes the structural switch of an ACD into a folded APT to facilitate the aptamer-ligand binding. The disruption of the ACD can be detected by FRET.

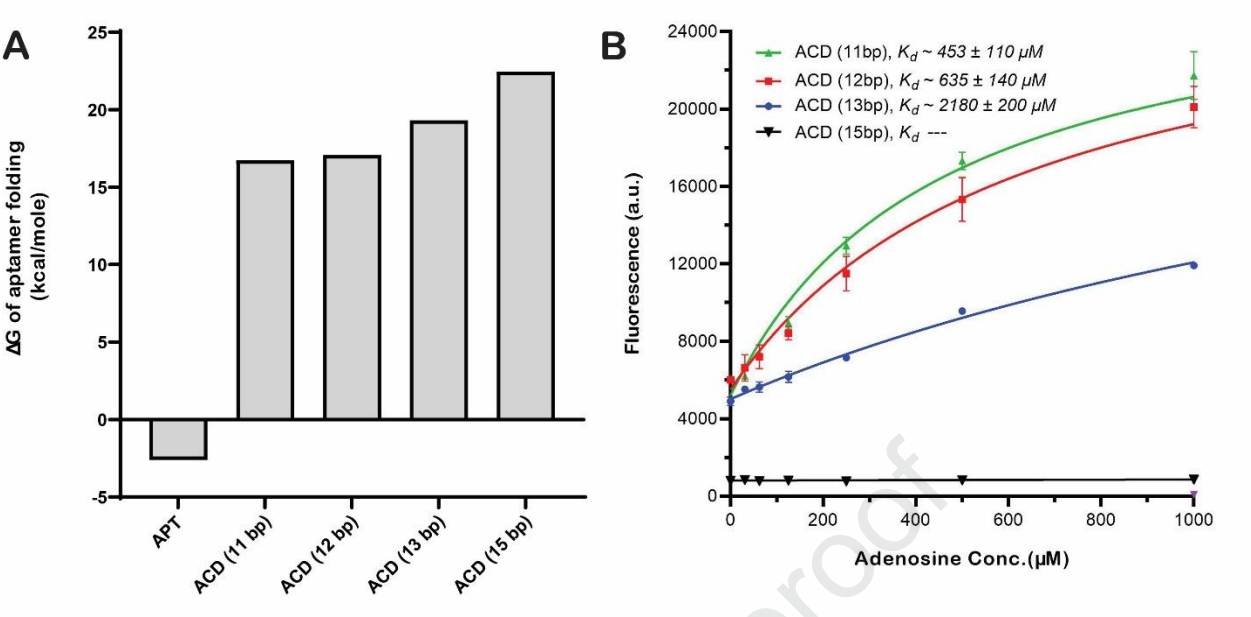

Figure 2. The length of an ACD for impacting the aptamer-ligand binding. (A) The folding energy of an aptamer and ACDs (11 – 15 bp). (B) The fluorescence measurement of various aptamer-complement duplexes for binding to adenosine and estimated apparent K_d values.

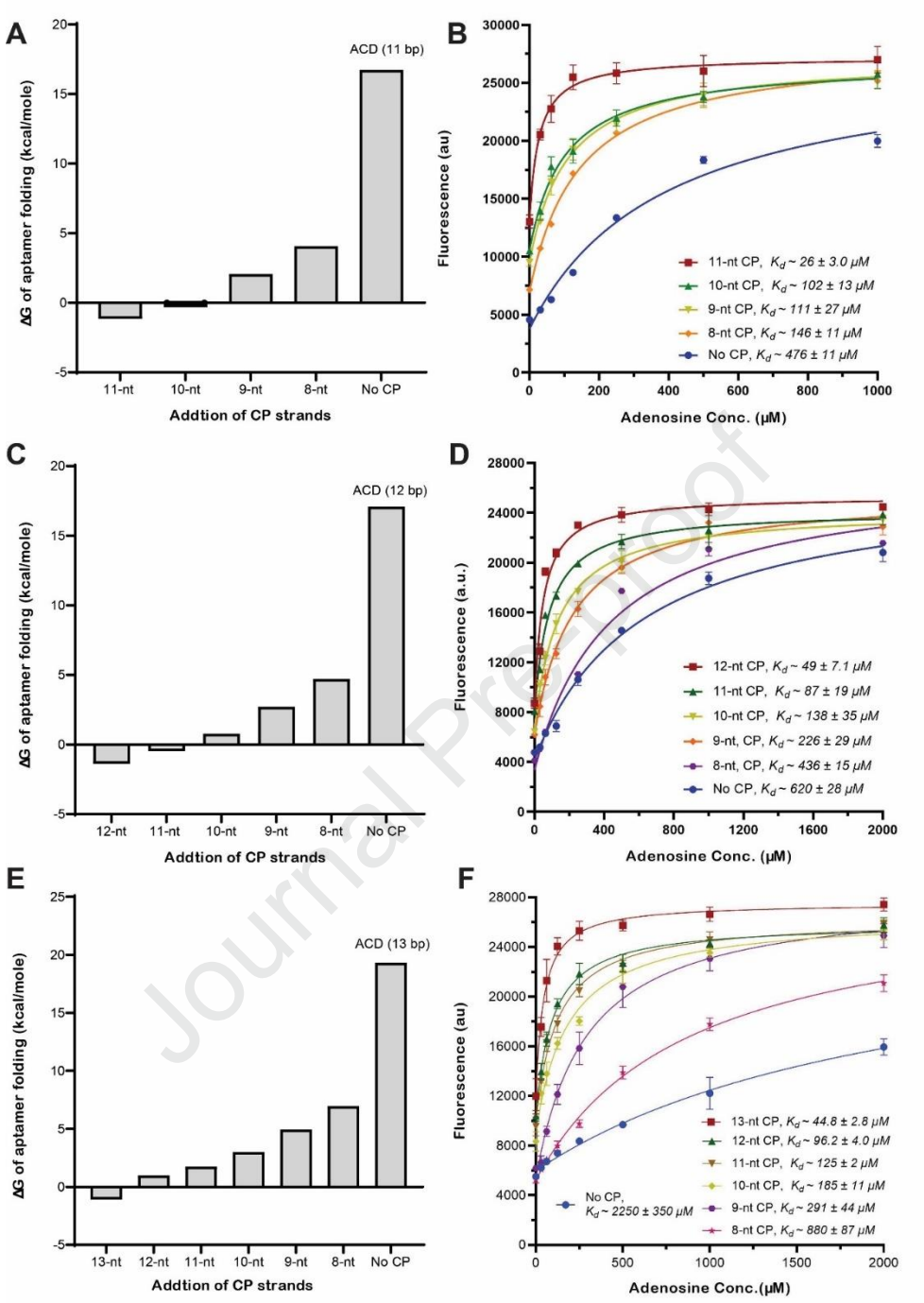

Figure 3. Evaluation of CP strands for affecting aptamer-ligand binding. The change of aptamer folding energy of ACDs and K_d measurement for 11-bp ACD (A, B),12-bp ACD (C, D) and 13-bp ACD (E, F).

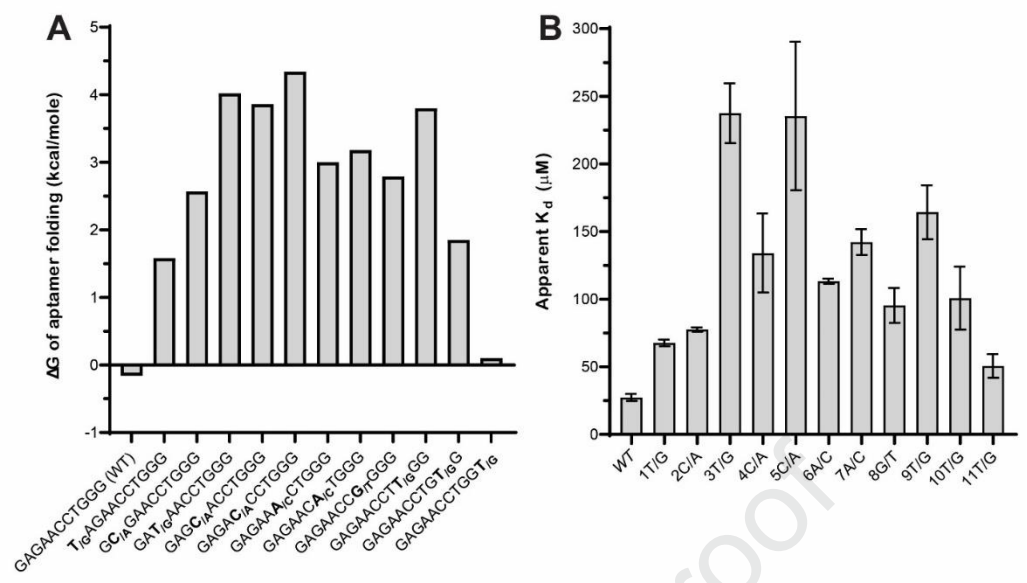

Figure 4. Single-nucleotide mutations (SNMs) for affecting competitive aptamer switch. (A) Aptamer folding energy for 11-bp ACD with the addition of CP strands containing SNMs. Calculation is set at 0.137 M Na^+ and 0 Mg^{2+}. (B) Experimental K_d measurement for CP strands containing SNMs.

ASSOCIATED CONTENT

Supporting Information.

Titration of Mg^{2+} (Figure S1), native aptamer binding to an adenosine (Figure S2), NAD^{+} binding to an ACD (Figure S3), CP to ACD ratio (Figure S4), kinetics profile of aptamer switch (Figure S5), raw titration curves for SNMs (Figure S6), DNA sequences (Table S1-3), thermal annealing program (Table S4), (PDF).

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Author Contributions

D.P. and S.W.O contributed equally to this manuscript. The manuscript was drafted by D.P. and J.F and was revised by all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Highlights

- Aptamer-complement duplex affects the binding affinity with increased K_d to a target.
- Competitive hybridization facilitates aptamer switches to decrease K_d of binding.
- An alternative approach to modulate the binding affinity and the sensitivity of aptamers.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: