

Light-up split aptamers: binding thermodynamics and kinetics for sensing

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Abstract

Due to their programmable structures, many aptamers can be readily split into two halves while still retaining their target binding function. While split aptamers are prevalent in the biosensor field, fundamental studies of their binding are still lacking. In this work, we took advantage of the fluorescence enhancement property of a new aptamer named OTC5 that can bind to tetracycline antibiotics to compare various split aptamers with the full-length aptamer. The split aptamers were designed to have different stem lengths. Longer stem lengths showed similar dissociation constants (K_d) to the full-length aptamer, while a shorter stem construct showed a 85-fold increase in K_d . Temperature-dependent fluorescence measurement confirmed lower thermostability of split aptamers. Isothermal titration calorimetry indicated that split aptamer binding can release more heat but have an even large entropy loss. Finally, a colorimetric biosensor using gold nanoparticles was designed by pre-assembling two thiolated aptamer halves, which can then link gold nanoparticles to give a red-to-blue color change.

Introduction

Aptamers are highly versatile for the development of biosensors, since they have predictable secondary structures, and their binding affinity can be rationally regulated by designing DNA base pairing interactions.¹⁻⁴ Some representative examples include aptamer beacons⁵ and structure-switching aptamers,⁶ both of which take advantage of additional DNA base pairing interactions that are regulated by aptamer binding. Splitting an aptamer into two halves is another popular way to achieve a range of signaling methods^{7, 8} from fluorescence,⁹ colorimetric,¹⁰ to Raman-based detection.¹¹ In a typical design, an aptamer is split from two base paired regions and the two halves would not bind to each other until a target molecule is added. Binding of the two split aptamer halves would bring them from infinitely far away to close proximity, resulting in a larger signal change compared to typical full-length aptamers.

Previous work on split aptamers has focused mainly on sensor design,^{7, 8, 12-15} or other applications,¹⁶ but fundamental binding properties of split aptamers versus full-length aptamers have yet to be studied.¹⁷ For example, it is perceived that split aptamers would suffer from a lower binding affinity due to disfavored entropy contributions, but the quantitative effects remain unknown. Understanding of these properties would be valuable for rational design of split aptamer biosensors.

Part of the reason for the lack of systematic binding assays is attributable to difficulties associated with small molecule aptamer binding assays.¹⁸⁻²⁰ For a systematic study to test many sequences, it is expensive to synthesize fluorescently labeled DNA. Recently, a few aptamers have been reported to show binding-induced fluorescence changes.²¹⁻²⁵ In those examples, target molecules are intrinsically fluorescent and the fluorescence intensity is either enhanced or quenched upon aptamer binding. We aimed to take advantage of such fluorescence to perform binding assays to understand split aptamers. One such example is the OTC5 aptamer that can bind to tetracycline antibiotics.^{22, 25, 26} OTC5 has a similar binding to a few tetracycline antibiotics. In this work, using fluorescence spectroscopy and isothermal titration calorimetry (ITC), we measured both the thermodynamic and kinetic properties of the full-length and split OTC5 aptamers. Based on our understanding and optimization, we also showed a way of colorimetric sensing using split aptamers and gold nanoparticles (AuNPs).

Materials and Methods

Chemicals. All DNA samples were purchased from Integrated DNA Technologies (Coralville, IA). Doxycycline and other chemicals used for the selectivity tests were purchased from Sigma-Aldrich. All buffers were from Mandel Scientific (Guelph, ON, Canada).²²

Fluorescence spectroscopy. Fluorescence experiments were performed on an Eclipse fluorescence spectrometer. A solution of 1 μM doxycycline was prepared in 10 mM MES pH 6 buffer with 2 mM MgCl_2 . OTC5a and the different stem lengths of OTC5b were pre-annealed in the same buffer for 5 min at 90°C followed by cooling slowly to room temperature. The annealed split aptamers were then titrated into the doxycycline solution up to a final concentration of 2 μM aptamer. The fluorescence was then read immediately after each addition using an excitation wavelength of 370 nm and emission wavelength of 535 nm. For temperature-dependent measurement, the experiment was performed in a Varian Eclipse fluorometer with a temperature control unit. For kinetic studies, 200 nM doxycycline was used.

Isothermal titration calorimetry (ITC). ITC was performed using a MicroCal ITC200 instrument. Data analysis was done using the accompanying Origin software. ITC was carried out at 20°C. 10 μM aptamer and 150 μM doxycycline were used for these experiments. The full length OTC5 aptamer or split OTC5a with either OTC5b4/5 or OTC5b7/5 was preannealed for 5 min at 90°C then allowed to cool slowly to 20°C before loading. All solutions were prepared using 20 mM MES pH 6 with 50 mM NaCl and 1 mM MgCl_2 .

UV-vis absorption spectroscopy. Absorbance values were obtained using an Agilent 8453 UV-vis spectrometer. Samples were collected using a absorbance range from 300-800 nm in triplicate measurements.

Synthesis of 55 nm AuNPs. Sodium tetrachloroaurate (121 mg) was first dissolved into H_2O (1 mL) and then added to remaining H_2O (999 mL), which was stirred and heated until boiling. Once boiling, sodium citrate (115 mg) was added and boiling was maintained for 15 min and then removed from heat to cool to room temperature. Continuous stirring was maintained throughout.

Colorimetric sensing. A solution of 1 nM of split aptamers, OTC5a and OTC5b5/6 were prepared in phosphate buffer pH 7.4 with 10 mM NaCl and 1 mM MgCl_2 . The aptamer halves were mixed in a 1:1 ratio, and annealed for 5 min at 95°C, followed by cooling to room temperature. A range of doxycycline concentrations (0.1-10 μM) were then added to the mixed aptamers and allowed to interact for 5 min. The aptamer/doxycycline mix was then added to 55 nm AuNPs in a 1:1 ratio,

and left to interact for 15 min on ice. The resulting samples were then characterized using UV-vis spectroscopy or a digital camera.

Results and Discussion

The split OTC5 aptamers

While numerous split aptamers have been reported,^{7, 8} we focused on the OTC5 aptamer in this work.²² The OTC5 aptamer has a similar affinity to tetracycline antibiotics such as tetracycline, oxytetracycline (OTC) and doxycycline ($K_d \sim 100$ nM).²² Binding to the OTC5 aptamer can induce a strong fluorescence increase of the tetracycline antibiotics.²⁵ OTC5 has a large loop and two short stems connecting them. Thus it is quite straightforward to split it through the two stems (Figure 1A). In the original aptamer, the two stems have 7 and 3 base pairs (bps), respectively. Since the 3 bp stem is in a hairpin, it also has a high stability.^{27, 28} After splitting, however, a 3 bp duplex is unlikely to be stable enough and thus we extended it by two bases to make a total of five base pairs in that stem (Figure 1A). Of the two split halves, one was named OTC5a and the other OTC5b. To have a systematic understanding, we fixed the OTC5a sequence but varied OTC5b to form different numbers of bps. The OTC5b shown in Figure 1A can form 7 and 5 bps in the two stems, respectively, and thus it was named OTC5b7/5. We also used a few other shorter OTC5b sequences such as OTC5b5/6, OTC5b4/6 and OTC5b5/4.

The main method used in this work is fluorescence spectroscopy. The fluorescence spectrum of the OTC5 full length aptamer was first measured. Doxycycline was chosen as the target molecule for these studies due to its better stability compared to tetracycline and oxytetracycline.²⁹ Doxycycline (1 μ M) was dissolved in MES pH 6.0 buffer with 2 mM MgCl₂. MgCl₂ is necessary to facilitate the binding of the aptamer as well as enhance the fluorescence signal.²⁵ The fluorescence spectrum was measured by exciting at 370 nm and emission was collected from 430 nm to 650 nm, both before and after the addition of 2 μ M OTC5 (Figure 1B). After the addition of 2 μ M OTC5, the fluorescence peak at 535 nm showed an 8-fold increase. This fluorescence enhancement was visible by eye under UV excitation in a dark room (Figure 1C).

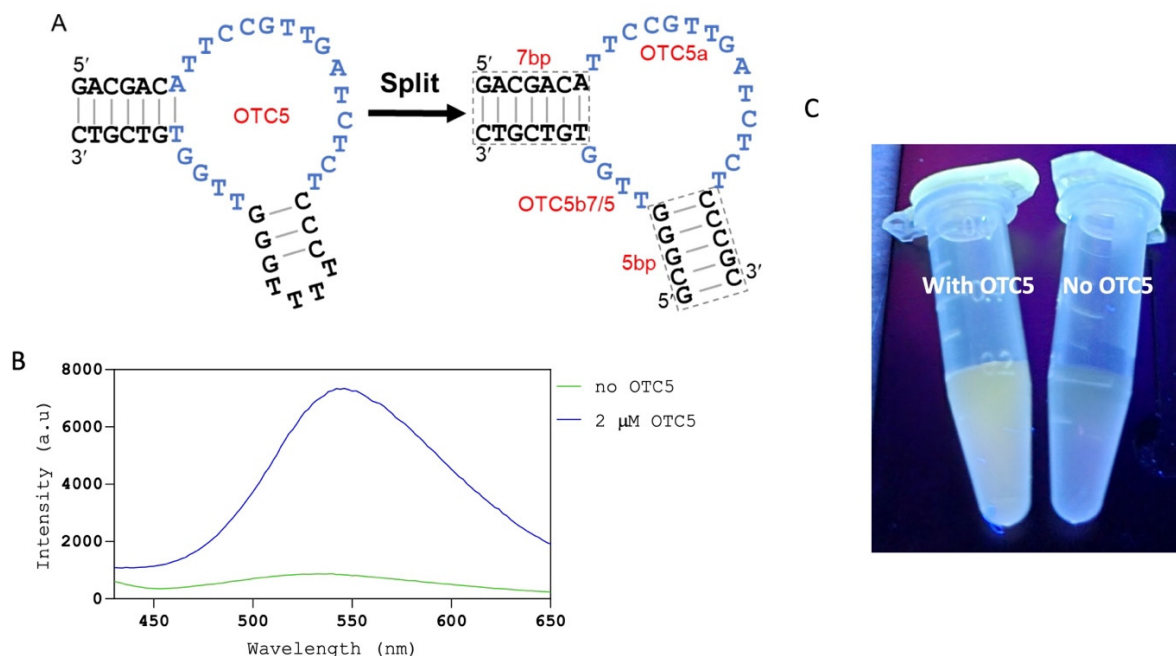


Figure 1. (A) The full-length OTC5 aptamer and the strategy of splitting. In this study, the OTC5a strand was fixed and the length of OTC5b strand varied to tune the number of base pairs. In the current figure, the two stems have 7 and 5 base pairs, respectively. (B) Fluorescence spectra of 1 μM doxycycline in the absence and presence of 2 μM of the full length OTC5 aptamer in 10 mM MES pH 6.0 buffer with 2 mM Mg^{2+} . (C) A photograph showing fluorescence enhancement of 2 μM doxycycline in the presence and absence of 5 μM C5 in the same buffer as (B).

Fluorescence change and binding affinity at different stem lengths

To quantitatively measure the effect of split aptamer on the binding affinity, we titrated the aptamers into their targets and monitored the fluorescence change. For the split OTC5 aptamers with various stem lengths, the OTC5b7/5 was the closest in binding to the full-length aptamer with a K_d of 0.55 μM (Figure 2). For comparison, the full-length has a fitted value of 0.27 μM . By shortening the stems to 5/6, the K_d increased to 1.8 μM . The signal of the 4/6 and 5/4 aptamers failed to saturate, and accurate K_d values cannot be obtained. Overall, this experiment showed that their ability to bind doxycycline was deteriorating by shortening the stems. Thus, the stem length

is very important to tune the binding affinity of split aptamers. Shorter stems can better disrupt the initial aptamer complex, but the overall binding ability is also more compromised.

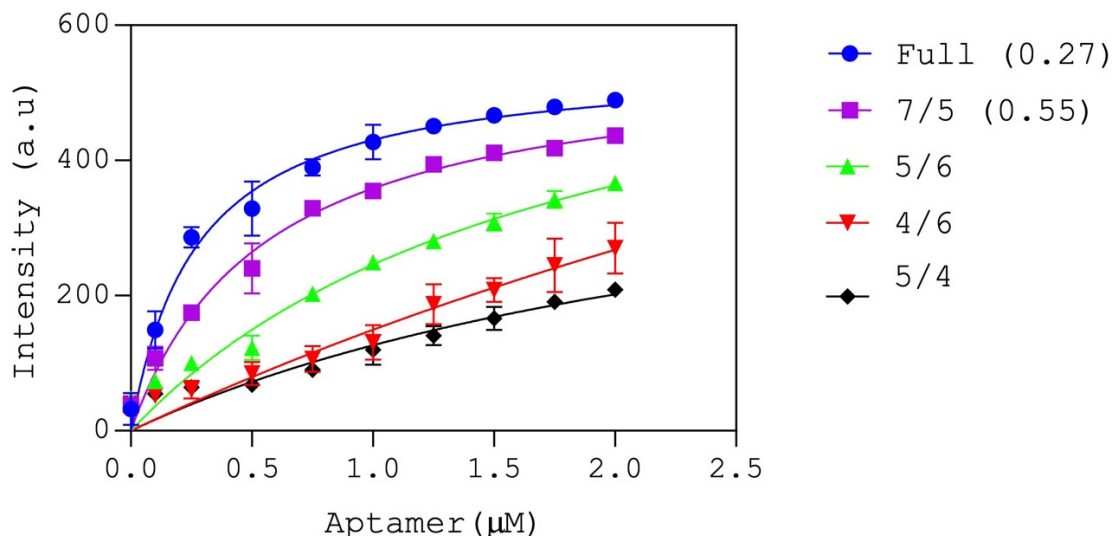


Figure 2. Fluorescence titration of each split aptamer into 1 μ M doxycycline. Fitted K_d values in μ M are in brackets. Split aptamers were pre-annealed at 90°C for 5 min and allowed to cool to room temperature before measurements.

Temperature-dependent binding

For a typical binding reaction, it is expected that increasing temperature would weaken binding due to increased contributions from the entropy term. To understand the temperature range for split aptamers, we first mixed aptamers with their targets and then gradually increased temperature. For the full-length OTC5, we observed two stages of fluorescence dropping. The first transition occurred below 20°C, and we attributed it to weak nonspecific association between doxycycline and the aptamer (Figure 3A, blue trace).³⁰ Then, relatively stable fluorescence was observed until 30°C, after which, another large fluorescence drop was observed, which was assigned to the dissociation of the aptamer complex. The melting temperature (T_m) of this step was calculated to be 34°C. At 40°C, the fluorescence dropped to the background level.

For the split OTC5 aptamer (Figure 3A, red trace), fluorescence drop started from 18°C, and the T_m was fitted to be 23°C. Therefore, in this case, splitting dropped the T_m by 11°C, despite that additional two base pairs were added to one of the stems. Therefore, splitting the aptamer has

decreased the thermostability of the binding complex. This can again be rationalized by an even larger entropy loss for the split aptamer upon target binding.

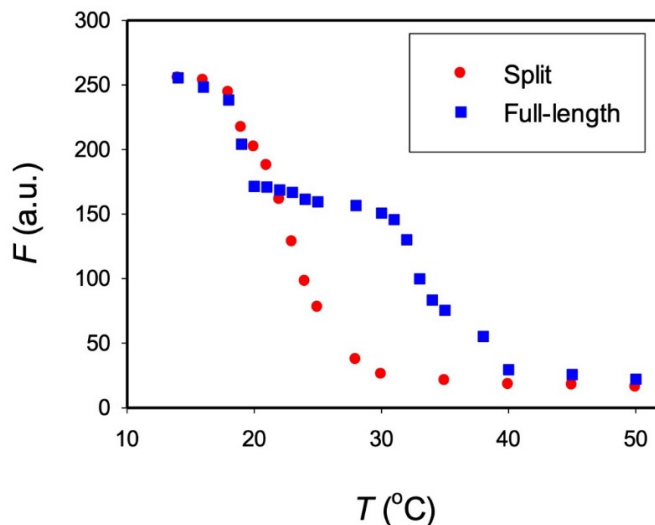


Figure 3. Temperature-dependent fluorescence of 1 μM doxycycline in the presence of 2 μM of the full-length or the split (5/6 bp) OTC5 aptamer. The measurement was performed in 10 mM MES buffer pH 6.0, 1 mM MgCl_2 and 50 mM NaCl.

Thermodynamics of binding measured using ITC

To further understand the thermodynamics of binding, ITC was performed on the full-length aptamer and two split aptamers with different stem lengths: OTC5b5/4 and OTC5b7/5 (Figure 4). ITC can provide full thermodynamic information of aptamer binding.³¹ All the aptamers showed exothermic peaks, and the fitted thermodynamic parameters are shown in Table 1. The original full length aptamer and the OTC5b7/5 split aptamer differed in K_d values by only three-fold. In addition, their enthalpy and entropy changes were also similar, indicating a similar binding to the target. However, when the stem was shortened to 5/4, the K_d was 85-fold higher. Thus, for split aptamers with long stems, likely the initial state was already hybridized and its binding to the target was similar to the full-length aptamer.

It was interesting to note that the split aptamers released even more heat or had a more negatively enthalpy change. This can be understood from that the split aptamer had two separate pieces. When they hybridize to form a full aptamer, a lot of hydrogen bonds were formed and they contributed to the heat release. The major factor for the decreased binding affinity was from the

entropy term, changing from -64.5 cal/mol·K for the full-length aptamer to -175 cal/mol·K to the split aptamer. Going from a split aptamer to a single binding complex resulted in a much larger entropy loss.

Based on the ITC data and the previous temperature-dependent fluorescence study, at low temperature, when the two halves of a split aptamer are already hybridized even in the absence of a target molecule, the binding performance of split aptamer and full-length aptamers are expected to be similar. However, split aptamers are more susceptible to high temperature due to a very large entropy loss.

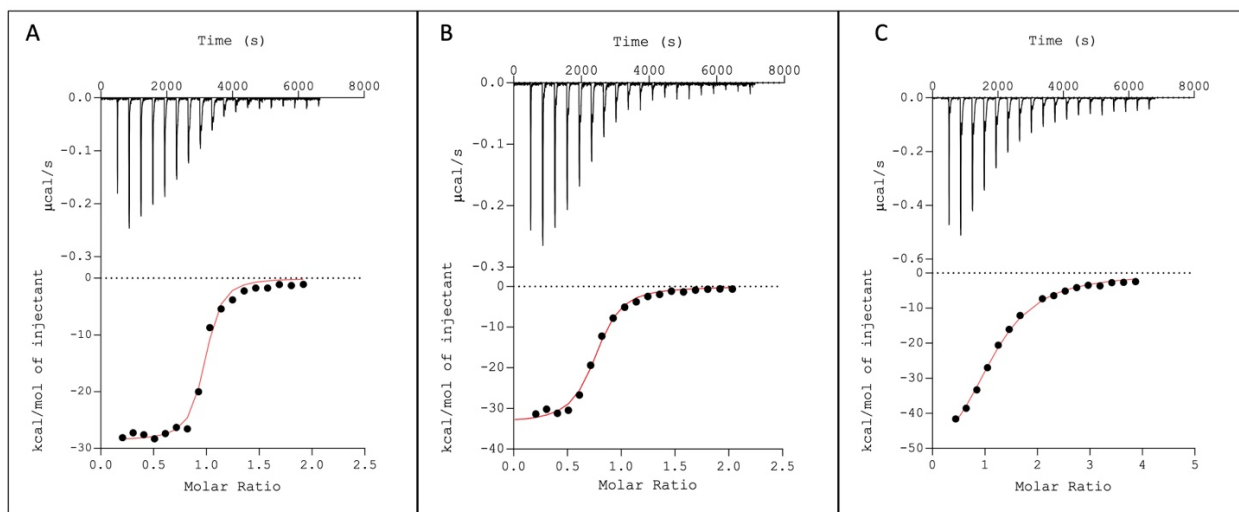


Figure 4. ITC traces of (A) the full length OTC5, (B) OTC5a+OTC5b7/5 aptamer, and (C) OTC5a+OTC5b5/4. All aptamers were pre-annealed for 5 min at 90°C and allowed to cool to 20°C before loading. Experiments were also carried out at 20°C.

Table 1. Fitted thermodynamic values of ITC experiments.

Aptamer	Target	<i>N</i>	<i>K_d</i> (μM)	<i>ΔH</i> (cal/mol)	<i>ΔS</i> (cal/mol/K)
OTC5	Doxycycline	0.95 ± 0.01	0.06 ± 0.02	-2.9 ± 0.6	-64.5
Split OTC5b7/5	Doxycycline	0.74 ± 0.01	0.19 ± 0.03	-3.4 ± 0.2	-83.8
Split OTC5b5/4	Doxycycline	1.1 ± 0.1	2.3 ± 0.2	-5.9 ± 0.2	-175

Binding kinetics comparison

After understanding the binding thermodynamics, we then studied the reaction kinetics. For split aptamers, there are two ways of mixing. First, we annealed split aptamers and then added doxycycline (Figure 5, black trace). Alternatively, one half of the aptamer can be mixed with doxycycline first followed by the addition of the other half (Figure 5, red and green traces). In this way, we also examined the hybridization kinetics. For biosensor applications, both types of mixing are important. When the split aptamer was annealed prior to addition, the fluorescence increase was instantaneous to a maximum of 4-fold enhancement of doxycycline fluorescence. When the two strands were added separately, the fluorescence only showed a significant increase when the second strand was added, and the kinetics was still very fast. Therefore, in this particular case, using split aptamers did not show a large adverse effect in signaling kinetics.

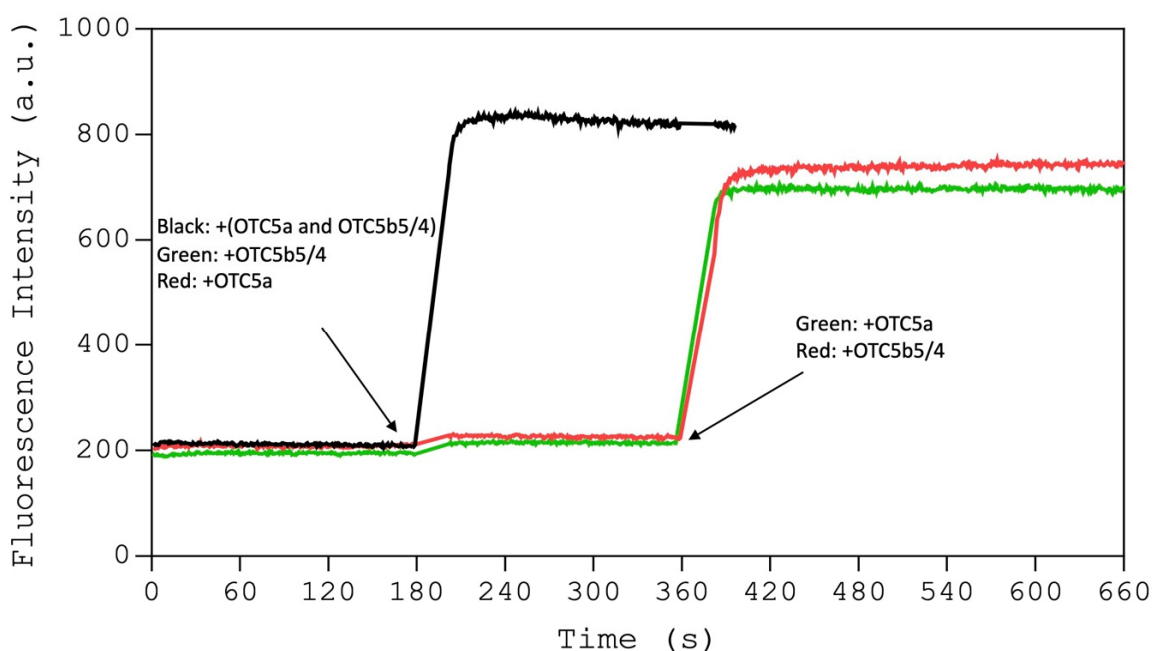


Figure 5. Kinetics of the split OTC5a and OTC5b5/4. Black: OTC5a and OTC5b5/4 were pre-annealed at 90°C and 1 μ M of the annealed solutions was added at 180 s. Red: 1 μ M OTC5a was added at 180 s followed by 1 μ M OTC5b5/4 at 360 s. Green: 1 μ M OTC5b5/4 was added at 180 s followed by 1 μ M OTC5a at 360 s. Excitation was at 370 nm and emission at 535 nm.

Doxycycline (200 nM) in MES pH 6.0 buffer with 1 mM MgCl₂ was read for 180 s to ensure a stable background before the addition of aptamers.

Split-aptamer assembly of gold nanoparticles

From this study, we understood the importance of the stem length and temperature on the binding of split aptamers. Based on our understanding, we designed a signaling method using split aptamers. We modified two split aptamer halves (OTC5a and OTC5b5/6) with a thiol group and inserted a 9-adenine spacer after the thiol. Interacting a combination of the two split aptamers with doxycycline can assemble the aptamer complex and expose the thiol groups, which can crosslink gold nanoparticles (AuNPs), prompt aggregation and change color from red to blue (Figure 6A).³² Otherwise, the thiolated aptamer halves would be individually adsorbed on the AuNPs, which can increase the colloidal stability and retain the original red color. These stem lengths were chosen since their doxycycline bound complex can form at room temperature of ~21°C based on our temperature-dependent study (Figure 3). Doxycycline is not expected to strongly interact with AuNPs to prevent its reaction with thiolated DNA.³³ A target-induced crosslinking sensor was previously reported by the Fan group,¹⁰ and our design here was different since the AuNPs were not pre-functionalized.

To confirm binding of the thiolated split aptamers, we still used the fluorescence titration method, and fluorescence increase was indeed observed upon adding the aptamers to oxytetracycline (Figure S1). The doxycycline-induced aggregation was then confirmed visually in Figure 6B using 55 nm AuNPs, where visible aggregation was seen after the addition of higher than 1 μM doxycycline, displaying a blue color compared to the red of the original AuNPs. The color change was quantified using UV/vis spectroscopy (Figure 6C), where increasing doxycycline concentrations showed increased aggregation indicated by the peak broadening in the 600 nm to 800 nm region and also overall decrease in peak intensity. Using extinction values of 700 nm and 519 nm, a peak ratio demonstrates the impact of aggregation with respect to doxycycline concentrations (Figure 6D). A detection range between 0.1 μM and 1 μM doxycycline was seen before aggregation became more predominant.

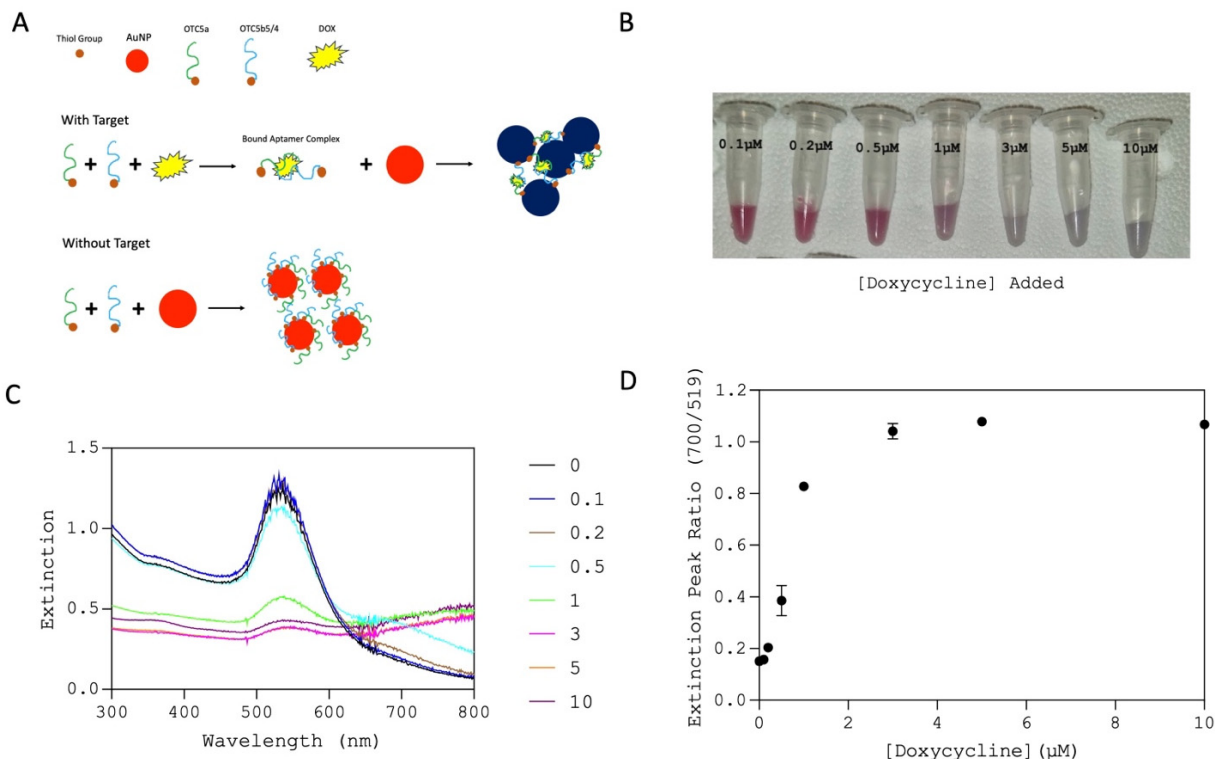


Figure 6. (A) A scheme of the colorimetric sensor based on split aptamers. (B) Photograph of aptamer, doxycycline and AuNP mixtures to visualize aggregation with color change of AuNPs. 10 nM split aptamers (OTC5a and OTC5b 5/6) were combined and annealed at 95°C for 1 min and cooled to room temperature. Doxycycline was added and then interacted with AuNPs to instigate an aggregation. (C) Extinction spectra of AuNPs mixed with the split thiolated OTC5 aptamers in the presence of varying concentrations of doxycycline in μ M. (D) Extinction ratio (at 700 nm/519 nm) of the data in (C) plotted against doxycycline concentration.

Conclusions

The OTC5 aptamer can bind to and enhance the fluorescence of doxycycline to such a considerable degree that it can be observed by the naked eye. In this work, we used this aptamer to study fundamental binding properties of split aptamers. The OTC5 aptamer was split into stems with different lengths to observe the effects on binding. Kinetics studies of the split aptamers showed that both components of the split aptamer are necessary for detectable fluorescence enhancement, but the order of addition had little effect. The enhancement in each case was also instantaneous. However, splitting the full-length aptamer does decrease binding affinity, except when long binding stems were designed, in which case the initial state of the aptamer is still a full aptamer

even without the target. ITC experiments further showed this loss of binding can be attributed to the greater loss in entropy. Finally, we designed a colorimetric signaling method by pre-assembling an aptamer complex in the presence of doxycycline, which can crosslink AuNPs to result in a color change. This is the first thorough study of the binding property of split aptamers, and it will be useful for researchers interested in using aptamers for the rational design of biosensors.

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References

1. J. Liu, Z. Cao and Y. Lu, *Chem. Rev.*, 2009, **109**, 1948–1998.
2. H. Yu, O. Alkhamis, J. Canoura, Y. Liu and Y. Xiao, *Angew. Chem. Int. Ed.*, 2021, **60**, 16800-16823.
3. E. M. McConnell, J. Nguyen and Y. Li, *Frontiers in Chemistry*, 2020, **8**, 434.
4. L. Wu, Y. Wang, X. Xu, Y. Liu, B. Lin, M. Zhang, J. Zhang, S. Wan, C. Yang and W. Tan, *Chem. Rev.*, 2021, **121**, 12035–12105.
5. J. J. Li, X. Fang and W. Tan, *Biochem. Biophys. Res. Commun.*, 2002, **292**, 31-40.
6. R. Nutiu and Y. Li, *J. Am. Chem. Soc.*, 2003, **125**, 4771-4778.
7. M. Debiais, A. Lelievre, M. Smietana and S. Müller, *Nucleic Acids Res.*, 2020, **48**, 3400-3422.
8. X. Qi, X. Yan, Y. Zhao, L. Li and S. Wang, *TrAC, Trends Anal. Chem.*, 2020, **133**, 116069.
9. M. N. Stojanovic, P. de Prada and D. W. Landry, *J. Am. Chem. Soc.*, 2000, **122**, 11547-11548.
10. F. Li, J. Zhang, X. N. Cao, L. H. Wang, D. Li, S. P. Song, B. C. Ye and C. H. Fan, *Analyst*, 2009, **134**, 1355-1360.
11. C. Zhou, Z. Yu, W. Yu, H. Liu, H. Zhang and C. Guo, *Microchimica Acta*, 2019, **186**, 251.
12. H. Yu, J. Canoura, B. Guntupalli, X. Lou and Y. Xiao, *Chem. Sci.*, 2017, **8**, 131-141.
13. A. Chen, M. Yan and S. Yang, *TrAC, Trends Anal. Chem.*, 2016, **80**, 581-593.
14. H. Ye, Z. Yang, I. M. Khan, S. Niazi, Y. Guo, Z. Wang and H. Yang, *Critical Reviews in Food Science and Nutrition*, 2022, 1-12.

15. X. Liu, J. Song, R. Yang, S. Su, L. Zhang, Y. Huang and Q. Fan, *IEEE Sens. J.*, 2022, **22**, 12460-12472.
16. Q. Chen, M. Jian, H. Chen, B. Zhou, H. Shi, X. Yang, K. Wang and J. Liu, *CCS Chemistry*, 2021, **3**, 144-153.
17. R. Wang, Q. Zhang, Y. Zhang, H. Shi, K. T. Nguyen and X. Zhou, *Anal. Chem.*, 2019, **91**, 15811-15817.
18. M. McKeague, A. De Girolamo, S. Valenzano, M. Pascale, A. Ruscito, R. Velu, N. R. Frost, K. Hill, M. Smith, E. M. McConnell and M. C. DeRosa, *Anal. Chem.*, 2015, **87**, 8608-8612.
19. E. Daems, G. Moro, R. Campos and K. De Wael, *TrAC, Trends Anal. Chem.*, 2021, **142**, 116311.
20. Y. Zhao, K. Yavari and J. Liu, *TrAC, Trends Anal. Chem.*, 2022, **146**, 116480.
21. A. A. Shoara, S. Slavkovic, L. W. Donaldson and P. E. Johnson, *Can. J. Chem.*, 2017, **95**, 1253-1260.
22. Y. Zhao, S. Ong, Y. Chen, P.-J. Jimmy Huang and J. Liu, *Anal. Chem.*, 2022, **94**, 10175–10182.
23. D. E. Armstrong-Price, P. S. Deore and R. A. Manderville, *J. Agric. Food. Chem.*, 2020, **68**, 2249-2255.
24. A. A. Shoara and P. E. Johnson, *Aptamers*, 2022, **6**.
25. Y. Zhao, B. Gao, P. Sun, J. Liu and J. Liu, *Biosensors*, 2022, **12**, 717.
26. Y. Zhao, B. Gao, Y. Chen and J. Liu, *Analyst*, 2023, **148**, 1507-1513.
27. H. Zhang, F. Li, B. Dever, X.-F. Li and X. C. Le, *Chem. Rev.*, 2013, **113**, 2812-2841.
28. A. B. Bezerra, A. S. N. Kurian and C. J. Easley, *Anal. Chem.*, 2021, **93**, 198-214.
29. M. Hassani, R. Lázaro, C. Pérez, S. Condón and R. Pagán, *J. Agric. Food. Chem.*, 2008, **56**, 2676-2680.
30. R. Oehler, N. Polacek, G. Steiner and A. Barta, *Nucleic Acids Res.*, 1997, **25**, 1219-1224.
31. S. Slavkovic and P. E. Johnson, *Aptamers*, 2018, **2**, 45-51.
32. N. L. Rosi and C. A. Mirkin, *Chem. Rev.*, 2005, **105**, 1547-1562.
33. Y. Song, D. H. G. v. Eppinghoven, Y. Zhou, H. Zhang and J. Liu, *Can. J. Chem.*, 2023, **101**, 343-352.