

Label-free and Dye-free Fluorescent Sensing of Tetracyclines Using a Capture Selected DNA Aptamer

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Abstract

Tetracyclines are a group of important antibiotics with a common four-ring scaffold. While most tetracyclines are currently used only in animals, their leaching into the environmental and residues in food have caused health concerns. Aptamers are an attractive way to detect tetracyclines, and all previously reported aptamers for tetracyclines were obtained by immobilizing target molecules. In this work, we selected a few DNA aptamers by immobilizing the DNA library using oxytetracycline as a target. We obtained new aptamers with no overlapping sequences compared to the previously reported ones, and a representative sequence named OTC5 had a dissociation constant of 147 nM measured by isothermal titration calorimetry. Similar binding affinities were also observed with tetracycline and doxycycline. Since tetracyclines are fluorescent and their fluorescence intensity was enhanced by binding to the aptamers, a label-free and dye-free fluorescent biosensor was developed with a detection limit of 25 nM oxytetracycline. The sensor was able to detect targets in milk after extraction. Fluorescence polarization measurement showed that this aptamer is insensitive to sodium concentration but requires magnesium. Finally, a strand displacement biosensor was designed and it has a detection limit of 1.2 μ M oxytetracycline.

Introduction

The tetracyclines refer to a family of linearly fused four-ring antibiotics that inhibit bacterial protein synthesis by impeding aminoacyl-tRNA attachment to ribosomes.¹ In 1949, oxytetracycline (OTC, trade name terramycine) was among the first discovered antibiotics in the tetracycline family as a natural product isolated from soil. A few years later, tetracycline (TC) was discovered. Since they stain the bones and teeth, TC and OTC are currently used mostly in animals.² Due to incomplete metabolism, their traces can be found in animal tissues or excreted and released into the environment.^{3,4} Studies in OTC led to the discovery of doxycycline (DOX) with a longer half-life in the body and better absorption, which becomes a highly popular antibiotic to date.

TC is known to interact with various RNA with micromolar affinities including the 30S ribosomal RNA, tRNA and some ribozymes.⁵ Aptamers are single-stranded oligonucleotides that can selectively bind target molecules.⁶⁻⁸ An RNA aptamer for TC was reported by Berens et al. in 2001 with a K_d of around 100 nM.⁵ A subsequent study optimized the sequence and achieved an even lower K_d .⁹ By rational randomization of this RNA aptamer, Tickner et al. selected an aptamer for DOX.¹⁰ A motivation of selecting RNA aptamers is to design artificial riboswitches.¹¹

DNA aptamers are more stable and easier to modify compared to RNA aptamers,^{6-8, 12, 13} and thus are more attractive for biosensor development.^{6-8, 12} DNA aptamers for tetracyclines have also been reported. In 2008, the first DNA aptamer for OTC was reported by Niazi et al., where the best aptamer had a K_d of 10 nM and the aptamer had little binding to TC or DOX.¹⁴ The same group then selected another aptamer using different magnetic beads respectively coated with TC, OTC and DOX, and the resulting general aptamer was reported to bind all the three antibiotics.¹⁵ Using a gold nanoparticle based colorimetric assay, this general aptamer was truncated down to just 8 nucleotides.¹⁶ So far, all of these aptamers were obtained by immobilization of target molecules.^{5, 14, 15} Library-immobilized selection (also called capture SELEX) allows the use of unmodified free targets.¹⁷⁻²² Many aptamers with tight binding affinities have been selected using this method (e.g. sub- μ M K_d for cortisol, dopamine and serotonin).^{17-19, 23-30}

TC, OTC and DOX are fluorescent with a large Stokes shift.³¹ Their fluorescence could be enhanced by binding to the RNA aptamer, which was used for the characterization of aptamer binding.^{9, 10} This useful property has yet to be used for developing biosensors. In this study, new

DNA aptamers were selected using OTC as a target through library-immobilized SELEX. A resulting aptamer could bind OTC, TC and DOX with a similar affinity. A label-free and dye-free fluorescent biosensor was demonstrated using the intrinsic fluorescence of the tetracyclines, which outperformed the strand-displacement sensor using a covalently labeled fluorophore and quencher.

Materials and Methods

Chemicals. All of the DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The DNA library consisted of a 30-nucleotide randomized region flanked by primer binding sequences. See Table S1 for the DNA sequences. TC, OTC, DOX and other chemicals were from Sigma-Aldrich.

Preparation of OTC solutions. During the aptamer selection, solutions of 2 mM and 0.5 mM OTC were prepared in 1× selection buffer. For rounds 1-14, the selection buffer was 50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 5 mM KCl, 10 mM MgCl₂ with 2% dimethyl sulfoxide (DMSO). DMSO was included to increase the solubility of OTC. For rounds 15-20, the OTC concentration was lowered to 0.5 mM and 0.1 mM and thus the same buffer without DMSO was used. For each selection round, OTC was freshly weighed and dissolved to avoid its degradation.³² Similarly, TC and DOX solutions were also freshly prepared before use.

SELEX for OTC Aptamers. Selection of DNA aptamers was conducted using the library immobilization method as previously described.^{18,26} For each round of selection, the DNA library was first annealed with the biotinylated capture strand. A column loaded with streptavidin-coated agarose microbeads was washed 5 times with 250 μL of 1× selection buffer. The library was then loaded onto the microbeads and washed 10 times with the selection buffer. The last three washes were kept as a background reference. 750 μL of the target solution was then passed through the library and the target bound run off was collected, purified by a 3k molecular weight cut-off spin column and concentrated to around 60 μL. The spin column was centrifuged at 17530g for 12 times to wash out target and concentrate the DNA. The concentration of OTC was 2 mM from round 1 to round 14, 0.5 mM from round 15 to round 17, and 0.1 mM from round 18 to round 20. Polymerase chain reaction (PCR) was then used to amplify the eluted DNA and one of the primers contained a biotin label. After incubation of the PCR products with the streptavidin beads, they

were then denatured using 0.2 M NaOH to recover the single-stranded library for the next round of selection. The final library was analyzed using deep sequencing at McMaster University as described previously.²⁶

ITC. Isothermal titration calorimetry (ITC) was performed using a MicroCal VP-ITC.²⁶ 150 μ M of a target molecule was titrated into 9 μ M aptamer. Both the target and the aptamer were in the same selection buffer. Background heat of titrating antibiotics into the buffer was subtracted. Data analysis was done using the accompanying Origin software.

Fluorescence polarization. To measure fluorescence polarization change of OTC upon binding to its aptamer, 100 nM OTC was dissolved in 100 μ L buffer. The buffers were 50 mM Tris (pH 7.8) containing different concentrations of MgCl₂ and NaCl. To the OTC samples, various concentrations of OTC5 aptamer were titrated, and each experiment was repeated 3 times. To measure polarization (Tecan Spark microplate reader), the excitation wavelength was set at 370 nm, and the emission wavelength was 530 nm. The change in polarization relative to the sample without the aptamer (ΔP) was plotted. All the binding curves were fitted using $y = y_0 + a[C]/(K_d + [C])$, where $[C]$ is the concentration of the titrated aptamer, and a is the maximal signal change upon saturated binding.

Label-free and dye-free OTC detection. The experiments were performed on a Varian Eclipse fluorimeter with excitation wavelength set for OTC at 370 nm. The emission spectra were collected from 500 nm to 600 nm. To determine the K_d , 100 nM OTC, TC or DOX was dissolved in 500 μ L buffer (10 mM Tris HCl, pH 7.8, 60 mM NaCl, 2 mM MgCl₂, 1 mM KCl). The OTC5 aptamer was titrated such that the final volume increase was less than 10%. The solution was mixed during each titration step and allowed to equilibrate for 1 min before data collection. Similar methods were used for the measurement of OTC concentration dependent fluorescence without or with 2 μ M OTC5 aptamer.

Detection in milk. Milk (2% fat) was purchased from a local supermarket. Six 1.2 mL milk samples were first spiked with different concentrations of OTC. To extract antibiotics, 30 μ L HCl (1 M) was added to each milk sample and the samples were centrifuged at 14000 rpm for 15 min. The supernatants were then removed and diluted 100-fold in 1 \times Dulbecco's PBS buffer (pH 7.2) containing calcium and magnesium. 98 μ L of this solution was then added to each well in a 96-well plate. Two blanks of water and buffer were also added to the plate. The base fluorescence was

read using the Tecan microplate reader. Then, 2 μ L of 100 μ M OTC5 aptamer (in water) was added for a final of 2 μ M OTC5, and the fluorescence was immediately read.

Fluorescence biosensor based on strand displacement. 20 nM of the FAM-labeled aptamer and 40 nM of the quencher-labeled strand were first hybridized in the selection buffer by heating for 1 min at 95°C and cooled to room temperature. This hybridized solution was diluted to 10 nM in 1 \times selection buffer and was added to each well of a 96-well microplate. The fluorescence was monitored at 520 nm with 485 nm excitation. The background was run prior to the addition of the target to ensure a low background signal. Then various concentrations of OTC were added at the 5 min mark.

Results and Discussion

Target-immobilized aptamer selection. OTC has a -OH group at both the 5' and 6' positions (Figure 1A). Replacing the 5'-OH with a proton results in TC, while replacing the 6'-OH with a proton results in DOX. In this study, we used OTC as the target due to its similarity to these two analogs, and we hoped to obtain an aptamer that can bind all these three tetracyclines to develop a general biosensors. We performed DNA aptamer selection using the library-immobilization method.^{18, 26, 33} The DNA library contained 30 random nucleotides flanked by two constant primer binding regions. The primer binding regions can form a 9-base-pair stem (Table S1), thus folding the library to an overall hairpin structure. The library was first hybridized to a short complementary strand with a biotin tag for immobilization on streptavidin-coated agarose beads. The selected sequences can bind to OTC and promote the formation of the hairpin. Thus, they were released from the beads for PCR amplification.

The selection was performed for a total of 20 rounds with the OTC concentration gradually decreased from 2 mM to 0.1 mM. The resulting PCR amplicons from round 20 were deep sequenced. Around 30,000 sequences were obtained and they were processed using the Geneious software. We then took the most abundant 15 sequences (which together counted for 65.3% of the entire library), and aligned them with the help of Cluster Omega (Figure 1B). These 15 sequences can be grouped into four families. Family 1 is represented by sequence OTC1, which can fold into a secondary structure shown in Figure 1C. The regions in green covaried to form a base paired

stem, while the nucleotides between them are rich in pyrimidines. The rest of the sequences were also well aligned (highlighted in blue). The next family is represented by OTC5 and it has a very small hairpin with a 3 base pair stem and a 4-nt loop (Figure 1D). The loop is rich in thymine but also contains other nucleotides (see Family 2 alignment in Figure 1B). The other two families have lower abundance and were not further analyzed.

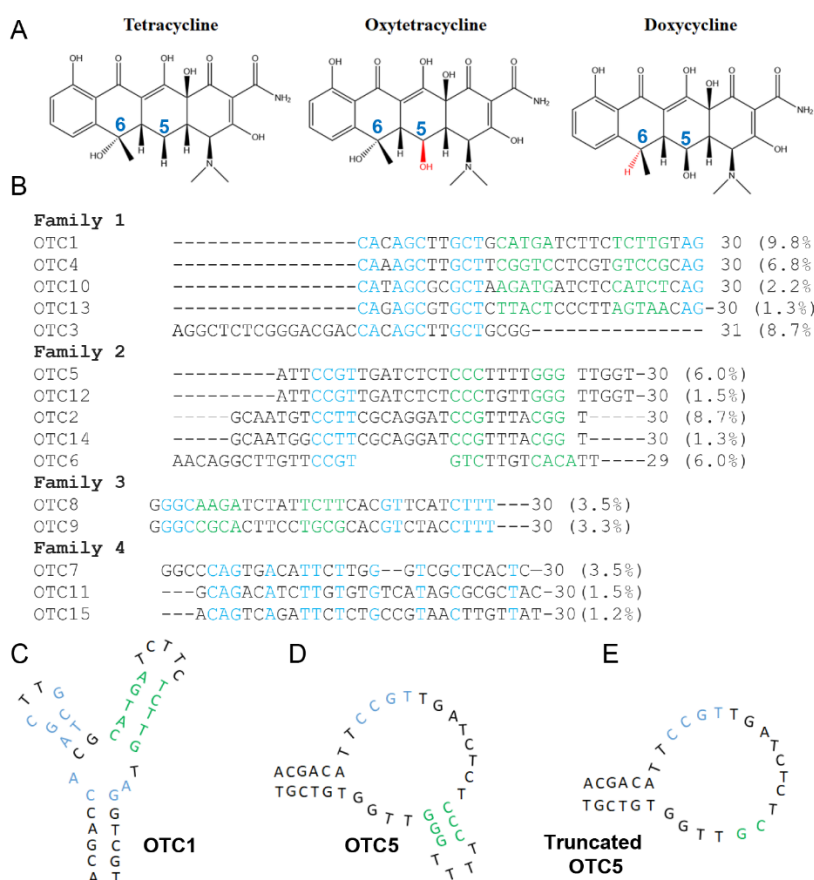


Figure 1. (A) The structures of the three tetracycline antibiotics used in this study. (B) Alignment of the most abundant 15 sequences that were divided into four families. Within each family, the nucleotides in green can base pair, while those in blue are identical. Mfold predicted secondary structures of (C) the OTC1 aptamer, (D) the OTC5 aptamer, and (E) a truncated OTC5 aptamer with the small hairpin removed.

We also compared our sequences with previously reported OTC DNA aptamers obtained from target-immobilized selections.^{14, 15} For example, we aligned the No. 4 aptamer¹⁴ against our

top sequences, but they do not have sequence homology (Figure S1). We also searched for the truncated 8-mer aptamer (CGGTGGTG),¹⁶ and no consensus was found with our top 10 sequences either. Therefore, what we obtained were new aptamers. Since we used unmodified OTC, aptamers can access all its surfaces for binding. For the immobilized OTC, part of the molecule became unavailable for aptamer binding and thus the resulting aptamers could bind differently.

Aptamer binding assay using ITC. To characterize binding of the aptamers, isothermal titration calorimetry (ITC) was used. ITC is a label-free technique that measures the heat of binding.³⁴⁻³⁶ For the sequences in Family 1, we first titrated OTC into the OTC1 aptamer (Figure 2A). The downward spikes indicated an exothermic reaction, and based on the integrated heat, a K_d of 0.6 μM was fitted. A mutant of OTC1 was also tested and no binding was detected (Figure S2), indicating the binding of OTC1 was specific. For the Family 2 sequences, we studied OTC5 (Figure 2B), and an even lower K_d (0.15 μM) was achieved. To confirm specific binding, an OTC5 mutant was tested by changing a fraction of the loop sequence from GTTG to TGGT, and this modification resulted in a loss of binding (Figure S3A). To confirm whether the small hairpin is important or not, we further tested a truncated version (Figure 1E), which had little heat release as well (Figure S3B). Thus, this small hairpin cannot be removed. The covariation of the stem region (the green nucleotides in Family 2, Figure 1B) suggests that it is a real hairpin structure. We also tested the binding of TC and DOX by OTC5 and they both showed a similar binding affinity (Figure 2C, D and Table 1). Based on the lower K_d of OTC5 than OTC1 and its ability to bind all the three tetracyclines, our subsequent studies focused on the OTC5 aptamer.

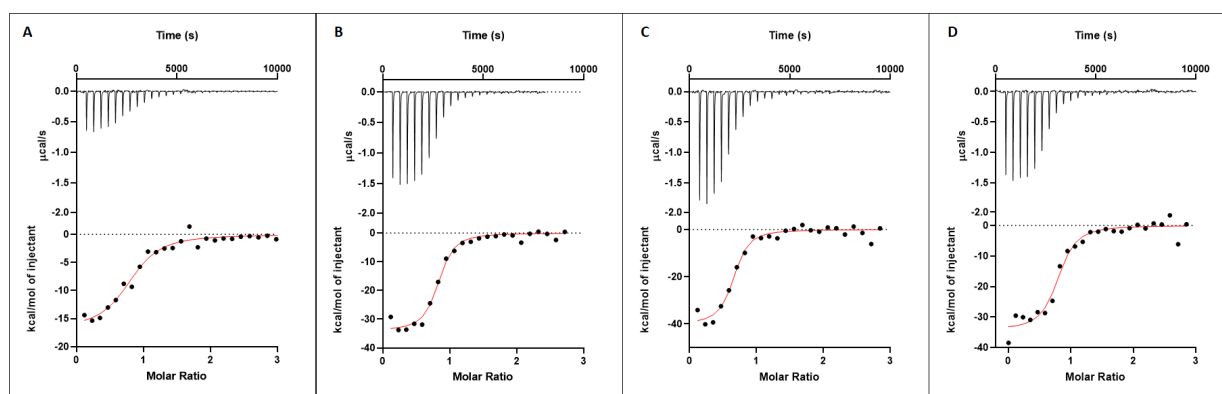


Figure 2. ITC traces and integrated heat of (A) titrating OTC into the OTC1 aptamer; and titrating (B) OTC, (C) TC and (D) DOX into the OTC5 aptamer. For each titration, 150 μM of target was titrated into 9 μM of DNA aptamer in the selection buffer at 25°C.

Table 1. Thermodynamic values of the aptamers based on ITC. Binding ratio (N), dissociation constant (K_d), enthalpy change (ΔH) and entropy change (ΔS) of the binding reactions are supplied.

| Aptamer | Target | N | K_d (μM) | ΔH (cal/mol) ($\times 10^4$) | ΔS (cal/mol/k) |
|---------|--------|-----------------|-------------------------|---|---------------------------|
| OTC1 | OTC | 0.81 ± 0.03 | 0.6 ± 0.1 | -1.7 ± 0.9 | -28.1 |
| OTC5 | OTC | 0.80 ± 0.03 | 0.15 ± 0.05 | -3.3 ± 1.6 | -79.3 |
| OTC5 | TC | 0.63 ± 0.02 | 0.18 ± 0.05 | -4.0 ± 1.9 | -104 |
| OTC5 | DOX | 0.77 ± 0.03 | 0.25 ± 0.06 | -3.4 ± 1.6 | -84.9 |

Aptamer binding assay using fluorescence polarization.

A unique feature of the tetracyclines is that they are fluorescent with a large Stokes shift in water.³¹ Such fluorescence is useful for measuring aptamer binding.^{9, 10} In the selection buffer, OTC has an emission peak at 530 nm when excited at 370 nm (Figure 3A). The intrinsic fluorescence of OTC makes it possible to use fluorescence polarization (FP) to study aptamer binding. FP measures the extent of fluorophore motion during the excited-state lifetime. Thus, aptamer binding can lead to increased FP. While FP is independent the concentration of fluorophores, the fluorescence intensity cannot be too low.^{37, 38} In our system, the coefficient of variation of FP was greater than 0.5 with 10 nM OTC and was less than 0.2 with 100 nM OTC. Therefore, we fixed the OTC concentration at 100 nM and then gradually titrated the OTC5 aptamer. In a buffer containing 300 mM NaCl and 10 mM MgCl₂, the fitted K_d value was 82 nM (Figure 3B, black trace), which was comparable with the 127 nM value from ITC. However, when the non-binding mutant was used, no change in the FP was observed (Figure 3B, blue diamonds). This experiment also confirmed binding of OTC by the aptamer.

We then used FP to explore the binding of OTC in different types and concentrations of salt, which is important for biosensors. We first kept 10 mM MgCl₂ and varied the NaCl concentration, where binding was slightly enhanced when NaCl was decreased (Figure 3B, K_d reached 45 nM when no NaCl was present). With fixed 100 mM NaCl, we then varied the concentration of MgCl₂ from 10 mM to 0. A similarly strong binding was observed when the Mg²⁺ concentration was higher than 1 mM, but when no Mg²⁺ was present, the binding was lost (Figure 3C). Therefore, a low concentration of Mg²⁺ was required for binding. Given the highly conjugated aromatic

structure of OTC, π - π stacking and hydrogen bonding could be important interaction mechanisms. In addition, Mg^{2+} is likely to mediate critical metal-bridged interactions.

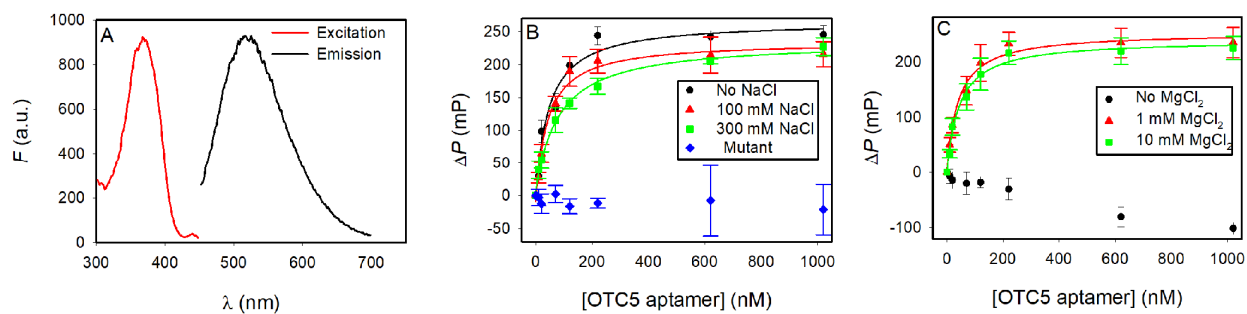


Figure 3. (A) Fluorescence excitation and emission spectra of 2.5 μ M OTC. (B) FP change measured by titrating the OTC5 aptamer into 100 nM OTC in the presence of different concentrations of NaCl, and all the samples contained 10 mM $MgCl_2$ in pH 7.8 Tris buffer. The mutant was measured in 10 mM $MgCl_2$ and 100 mM NaCl. (C) FP change measured with different concentrations of $MgCl_2$, and all the samples contained 100 mM NaCl. Bars are standard deviations from three independent measurements.

Label-free and dye-free sensing. While FP is useful for the characterization of aptamer binding, it is less so for the quantification of OTC, since FP is insensitive to the concentration of fluorophores. To achieve quantitative detection, we then resorted to binding-induced fluorescence intensity change. When the OTC5 aptamer was titrated into 100 nM OTC, the fluorescence gradually increased (Figure 4A), and we fitted the peak fluorescence to obtain a K_d of 98 nM (Figure 4B), which was comparable to the values obtained from ITC and FP. If the non-binding mutant was titrated, no fluorescence change was observed (Figure 4B). We also did the same experiment with TC (Figure 4C) and DOX (Figure 4D), and they also showed a similar fluorescence enhancement with a K_d of 264 nM TC and 145 nM DOX. The aptamer provided a more hydrophobic environment for the tetracyclines, which can inhibit non-radiative processes leading to enhanced fluorescence.

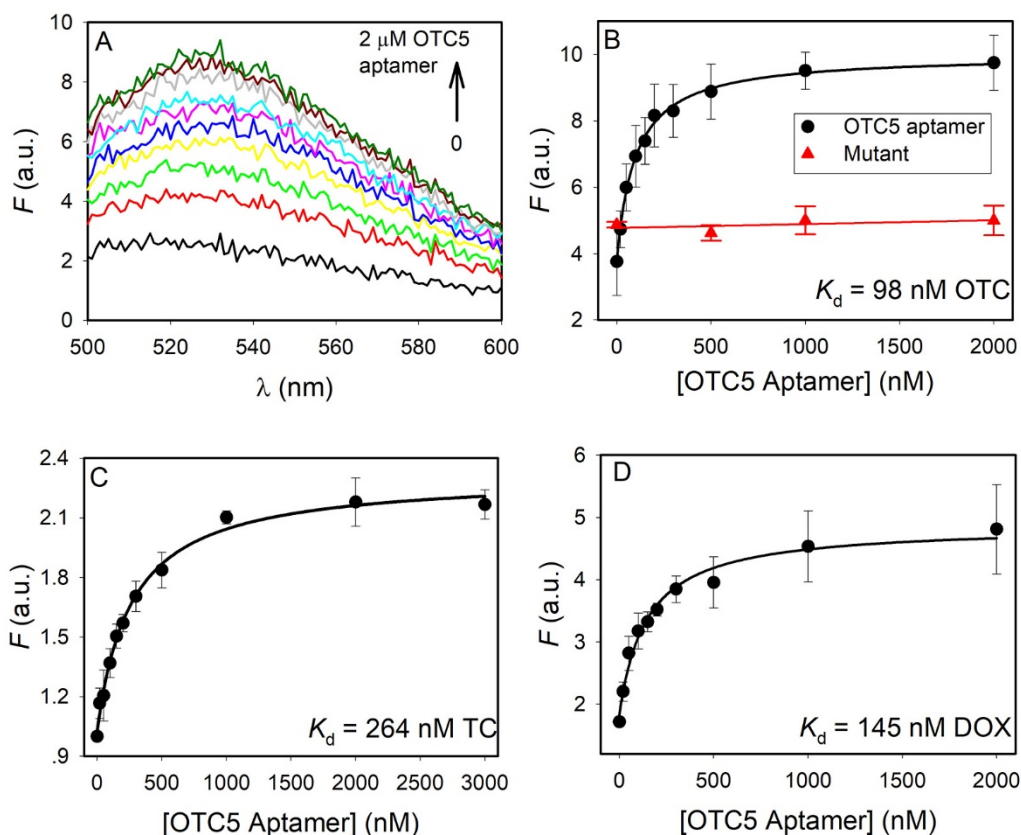


Figure 4. (A) Fluorescence spectra of 100 nM OTC in the presence of various concentration of the OTC5 aptamer with 370 nm excitation. Fluorescence intensities of 100 nM (B) OTC, (C) TC, and (D) DOX with increasing concentration of the OTC5 aptamer. The data were fitted to a one-site binding curve. In (B), the non-binding mutant was also tested.

Such a unique aptamer-induced fluorescence increase might be useful for detection. We can divide analytes into three types. First, none fluorescent molecules would have no fluorescence before or after aptamer addition. Second, most fluorophores would not be affected by the aptamer either. Thus, only the tetracyclines can show increased fluorescence (Figure 5A). Since no covalent labels or DNA staining dyes were used,^{39, 40} we called it a label-free and dye-free sensor. We measured the fluorescence of OTC as a function of its concentration in the absence and presence of $2 \mu\text{M}$ OTC5 aptamer (Figure 5B). A linear response was observed for both, and the sample with the aptamer had a 2.2-fold higher slope. Using the difference of the fluorescence intensity to build a calibration curve (Figure 5C), we determined the limit of detection (LOD) to be 25 nM ($3\sigma/\text{slope}$,

where σ is the background fluorescence variation without OTC). The increased fluorescence by the added OTC5 aptamer in the absence of OTC was attributed to the intrinsic fluorescence of DNA.⁴¹

Johnson and coworkers reported strong quenching of the intrinsic fluorescence of quinine and cocaine upon aptamer binding.⁴² While useful for the characterization of aptamer binding, fluorescence quenching is less favorable for biosensors. Manderville and coworkers used the intrinsic fluorescence of ochratoxin A for its detection, where signal-on sensing was achieved only when exciting the aptamer at 256 nm followed by energy transfer.⁴³ While many fluorogenic aptamers were reported,^{44, 45} their ligands are typically not considered as target molecules. To the best of our knowledge, this is the first time that the intrinsic fluorescence of tetracyclines was used for designing aptamer-based signal-on biosensors.

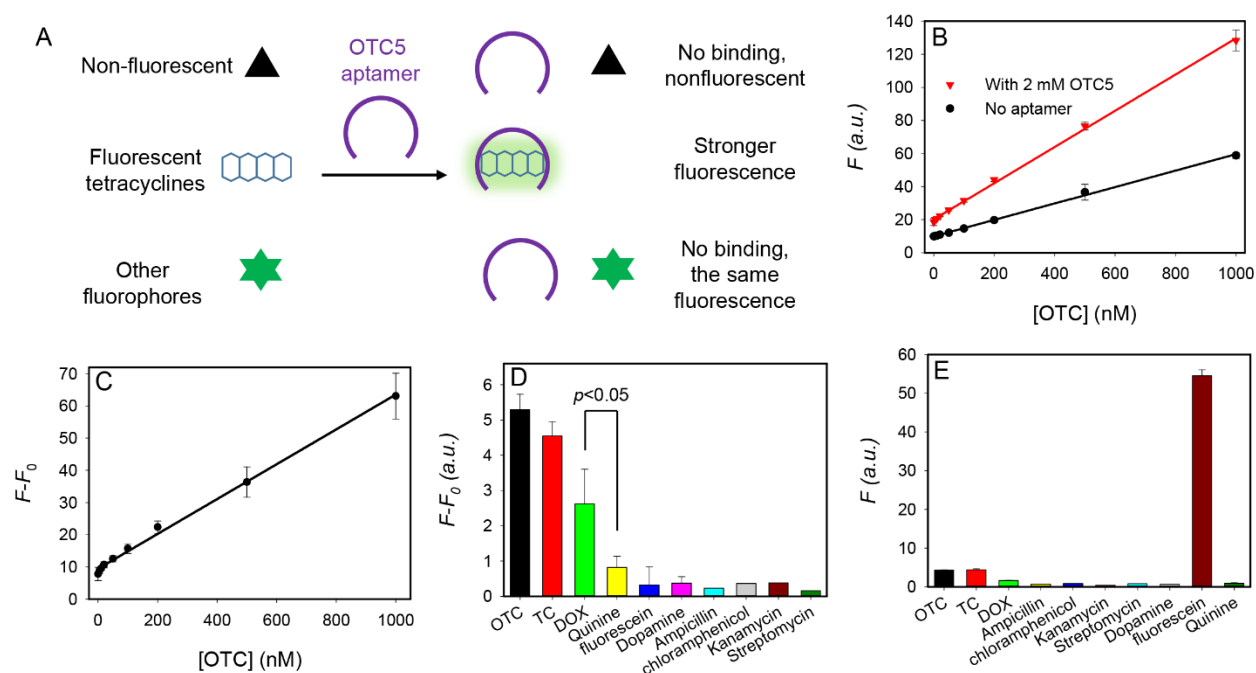


Figure 5. (A) A scheme showing the response of three types of molecules to the OTC5 aptamer, where only the tetracyclines show increased fluorescence upon aptamer binding. (B) Fluorescence as a function of OTC concentration without and with 2 μ M OTC5 in the selection buffer. (C) Fluorescence difference of OTC before and after adding 2 μ M OTC5 aptamer. (D) Fluorescence difference of various molecules before and after adding 2 μ M OTC5 aptamer. (E) Fluorescence

intensity at 530 nm of 100 nM molecules excited at 370 nm. The buffer was 10 mM Tris-HCl, pH 7.8, 60 mM NaCl, 2 mM MgCl₂, 1 mM KCl.

We then tested the selectivity of this sensor against a few other antibiotics (ampicillin, chloramphenicol, kanamycin and streptomycin). Since they are nonfluorescent, the signals were close to zero (Figure 5D). We also tested a few fluorescent compounds (fluorescein, dopamine, quinine). With 370 nm excitation, only fluorescein showed a strong 530 nm fluorescence emission (Figure 5E). Yet, its fluorescence was not affected by the OTC5 aptamer. As a result, the fluorescence difference before and after adding the aptamer was also close to zero (Figure 5D). Therefore, the background fluorescence in the sample would not affect the performance of the sensor. OTC, TC and DOX showed a similar response, and thus this sensor can detect them as a group.

Sensing of OTC in milk. We then tested this sensing method with milk samples. Milk was spiked with different concentrations of OTC. Since tetracyclines are known to bind to milk proteins,⁴⁶ they cannot be detected directly in milk. Thus, the samples were treated with HCl, centrifuged, and then diluted in Dulbecco's PBS buffer before reading the fluorescence. In the treated milk, the OTC5 aptamer also increased the fluorescence of the spiked OTC (Figure 6A). This sensor was able to sense OTC with a detection limit of 66 nM (Figure 6B).

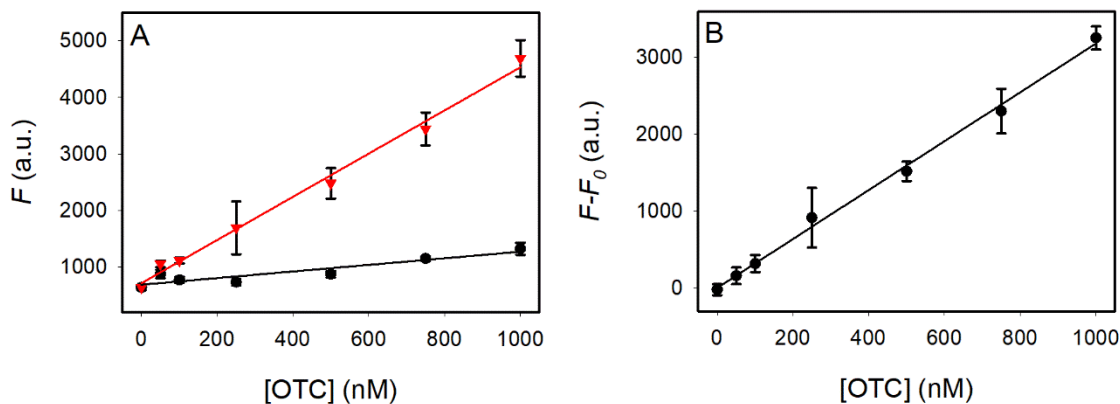


Figure 6. (A) Fluorescence intensity as a function of OTC concentration in spiked milk samples after extraction without and with 2 μ M OTC5 aptamer. (B) The fluorescence difference as a function of OTC concentration.

Labeled fluorescent sensing of OTC. Finally, we also designed a strand-displacement fluorescent sensor. The OTC5 aptamer was extended by six nucleotides on the 5'-end and labeled with a carboxyfluorescein (FAM) fluorophore. It was hybridized with a 12-mer quencher-labeled DNA.⁴⁷ In the presence of OTC, the aptamer would bind to it and released the quencher-labeled strand resulting in fluorescence enhancement (Figure 7A). At the excitation wavelength of FAM (485 nm), OTC was not excited and thus would not affect the detection.

The background of the sensor was monitored prior to the addition of OTC to ensure a stable sensor (Figure 7B). Then, various concentrations of OTC were added at the 5-min mark. After 5 min reaction, the fluorescence reached stable values for all samples. With 36 μM of target, over 20-fold increase in the fluorescence signal was observed after 20 min. A calibration curve was obtained showing a typical binding curve (Figure 7C). An apparent K_d of 12 μM was obtained. This value was much higher than what was obtained from the ITC experiments due to competition with the complementary strand.⁴⁷ Below 4 μM OTC, the response to was linear (Figure 7C, inset). Using this sensor, the limit of detection was calculated to be 1.2 μM OTC, which was nearly 50-fold worse than the above label-free and dye-free sensing method. The increased apparent K_d can explain the increased detection limit. For the selectivity test of the aptamer, 8 μM of different antibiotics were screened (Figure 7D). As expected, the tetracyclines showed a similar response, while the other antibiotics were silent. Overall, this strand-displacement sensor is more expensive yet less sensitive than the label-free and dye-free method.

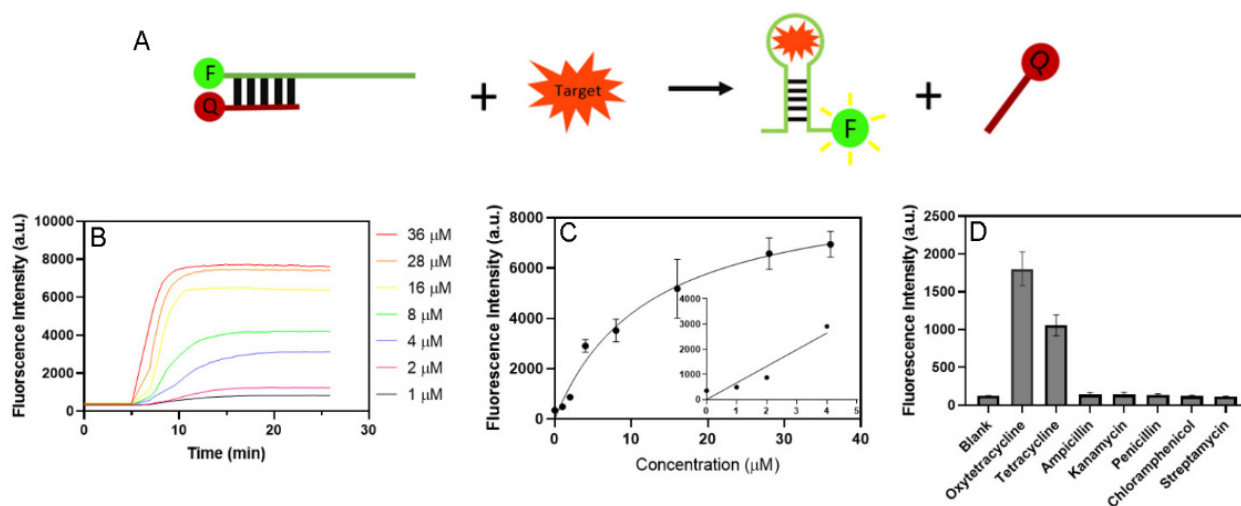


Figure 7. (A) Schematic of the strand-displacement biosensor with a covalently linked fluorophore and quencher. (B) Kinetics traces of the sensor in the presence of various concentrations of OTC in the selection buffer, and OTC was added at 5 min. (C) Fluorescence intensity as a function of OTC concentration. Inset: linear response at lower OTC concentrations. (D) Sensor selectivity against various antibiotics at 8 μM .

Conclusions

In this work, we used the library-immobilization method (so-called capture SELEX) to select new DNA aptamers for OTC. At least four families of aptamer sequences were obtained, and the OTC5 sequence was studied in detail due to its high binding affinity and generality to all the three tetracyclines. Similar binding to OTC, TC and DOX with K_d 's around 100 nM to 300 nM was observed using ITC, FP and fluorescence intensity measurements. These aptamers were different from the previously reported ones based on sequence alignment. The intrinsic fluorescence of these tetracyclines allowed for a highly sensitive and selective label-free and dye-free sensing method with a limit of detection of 25 nM OTC. Its performance was even better than the classic strand-displacement design with covalent labels, which had a limit of detection of 1.2 μM OTC. Milk was spiked with varying concentrations of OTC. After extraction, they were tested using the label-free biosensor. The intrinsic fluorescence of the tetracyclines is particularly useful to make it a model system to develop both labeled and label-free biosensors.

Acknowledgements

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Supporting Information

DNA sequences used in this work (Table S1), alignment of aptamer sequences with previous published aptamers (Figure S1), and control experiments for ITC (Figure S2, S3).

References

- (1) Chopra, I.; Roberts, M., Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232-260.
- (2) Granados-Chinchilla, F.; Rodríguez, C., Tetracyclines in Food and Feedingstuffs: From Regulation to Analytical Methods, Bacterial Resistance, and Environmental and Health Implications. *J. Anal. Meth. Chem.* **2017**, 2017, 1315497.
- (3) Daghri, R.; Drogui, P., Tetracycline Antibiotics in the Environment: A Review. *Environ. Chem. Lett.* **2013**, *11*, 209-227.
- (4) Mehlhorn, A.; Rahimi, P.; Joseph, Y., Aptamer-Based Biosensors for Antibiotic Detection: A Review. *Biosensors* **2018**, *8*, 54.
- (5) Berens, C.; Thain, A.; Schroeder, R., A Tetracycline-Binding RNA Aptamer. *Biorg. Med. Chem.* **2001**, *9*, 2549-2556.
- (6) Wu, L.; Wang, Y.; Xu, X.; Liu, Y.; Lin, B.; Zhang, M.; Zhang, J.; Wan, S.; Yang, C.; Tan, W., Aptamer-Based Detection of Circulating Targets for Precision Medicine. *Chem. Rev.* **2021**, *121*, 12035–12105.
- (7) McConnell, E. M.; Nguyen, J.; Li, Y., Aptamer-Based Biosensors for Environmental Monitoring. *Front. Chem.* **2020**, *8*, 434.
- (8) Yu, H.; Alkhamis, O.; Canoura, J.; Liu, Y.; Xiao, Y., Advances and Challenges in Small-Molecule DNA Aptamer Isolation, Characterization, and Sensor Development. *Angew. Chem. Int. Ed.* **2021**, *60*, 16800-16823.
- (9) Müller, M.; Weigand, J. E.; Weichenrieder, O.; Suess, B., Thermodynamic Characterization of an Engineered Tetracycline-Binding Riboswitch. *Nucleic Acids Res.* **2006**, *34*, 2607-2617.
- (10) Tickner, Z. J.; Zhong, G.; Sheptack, K. R.; Farzan, M., Selection of High-Affinity RNA Aptamers That Distinguish between Doxycycline and Tetracycline. *Biochemistry* **2020**, *59*, 3473-3486.
- (11) Wurmthaler, L. A.; Sack, M.; Gense, K.; Hartig, J. S.; Gamerdinger, M., A Tetracycline-Dependent Ribozyme Switch Allows Conditional Induction of Gene Expression in *Caenorhabditis Elegans*. *Nat. Commun.* **2019**, *10*, 491.
- (12) Wu, Y.; Belmonte, I.; Sykes, K. S.; Xiao, Y.; White, R. J., Perspective on the Future Role of Aptamers in Analytical Chemistry. *Anal. Chem.* **2019**, *91*, 15335-15344.
- (13) Debais, M.; Lelievre, A.; Smietana, M.; Müller, S., Splitting Aptamers and Nucleic Acid Enzymes for the Development of Advanced Biosensors. *Nucleic Acids Res.* **2020**, *48*, 3400-3422.
- (14) Niazi, J. H.; Lee, S. J.; Kim, Y. S.; Gu, M. B., Ssdna Aptamers That Selectively Bind Oxytetracycline. *Biorg. Med. Chem.* **2008**, *16*, 1254-1261.
- (15) Niazi, J. H.; Lee, S. J.; Gu, M. B., Single-Stranded DNA Aptamers Specific for Antibiotics Tetracyclines. *Biorg. Med. Chem.* **2008**, *16*, 7245-7253.
- (16) Kwon, Y. S.; Ahmad Raston, N. H.; Gu, M. B., An Ultra-Sensitive Colorimetric Detection of Tetracyclines Using the Shortest Aptamer with Highly Enhanced Affinity. *Chem. Commun.* **2014**, *50*, 40-42.

- (17) Nutiu, R.; Li, Y., In Vitro Selection of Structure-Switching Signaling Aptamers. *Angew. Chem. Int. Ed.* **2005**, *44*, 1061-1065.
- (18) Nakatsuka, N.; Yang, K.-A.; Abendroth, J. M.; Cheung, K. M.; Xu, X.; Yang, H.; Zhao, C.; Zhu, B.; Rim, Y. S.; Yang, Y.; Weiss, P. S.; Stojanović, M. N.; Andrews, A. M., Aptamer-Field-Effect Transistors Overcome Debye Length Limitations for Small-Molecule Sensing. *Science* **2018**, *362*, 319-324.
- (19) Yang, K.-A.; Barbu, M.; Halim, M.; Pallavi, P.; Kim, B.; Kolpashchikov, D. M.; Pecic, S.; Taylor, S.; Worgall, T. S.; Stojanovic, M. N., Recognition and Sensing of Low-Epitope Targets Via Ternary Complexes with Oligonucleotides and Synthetic Receptors. *Nat. Chem.* **2014**, *6*, 1003-1008.
- (20) Yu, H.; Luo, Y.; Alkhamis, O.; Canoura, J.; Yu, B.; Xiao, Y., Isolation of Natural DNA Aptamers for Challenging Small-Molecule Targets, Cannabinoids. *Anal. Chem.* **2021**, *93*, 3172-3180.
- (21) Yu, H. X.; Yang, W. J.; Alkhamis, O.; Canoura, J.; Yang, K. A.; Xiao, Y., In Vitro Isolation of Small-Molecule-Binding Aptamers with Intrinsic Dye-Displacement Functionality. *Nucleic Acids Res.* **2018**, *46*, e43.
- (22) Lyu, C.; Khan, I. M.; Wang, Z., Capture-Selelex for Aptamer Selection: A Short Review. *Talanta* **2021**, *229*, 122274.
- (23) Yang, K.-A.; Chun, H.; Zhang, Y.; Pecic, S.; Nakatsuka, N.; Andrews, A. M.; Worgall, T. S.; Stojanovic, M. N., High-Affinity Nucleic-Acid-Based Receptors for Steroids. *ACS Chem. Biol.* **2017**, *12*, 3103-3112.
- (24) Yang, W. J.; Yu, H. X.; Alkhamis, O.; Liu, Y. Z.; Canoura, J.; Fu, F. F.; Xiao, Y., In Vitro Isolation of Class-Specific Oligonucleotide-Based Small-Molecule Receptors. *Nucleic Acids Res.* **2019**, *47*, e71.
- (25) Gao, H. L.; Zhao, J. X.; Huang, Y.; Cheng, X.; Wang, S.; Han, Y.; Xiao, Y.; Lou, X. H., Universal Design of Structure-Switching Aptamers with Signal Reporting Functionality. *Anal. Chem.* **2019**, *91*, 14514-14521.
- (26) Huang, P.-J. J.; Liu, J., Selection of Aptamers for Sensing Caffeine and Discrimination of Its Three Single Demethylated Analog. *Anal. Chem.* **2022**, *94*, 3142-3149.
- (27) Luo, Y.; Wang, J.; Yang, L.; Gao, T.; Pei, R., In Vitro Selection of DNA Aptamers for the Development of Fluorescent Aptasensor for Sarcosine Detection. *Sens. Actuators B Chem.* **2018**, *276*, 128-135.
- (28) Tian, H.; Duan, N.; Wu, S.; Wang, Z., Selection and Application of ssDNA Aptamers against Spermine Based on Capture-SELEX. *Anal. Chim. Acta* **2019**, *1081*, 168-175.
- (29) Liu, Y.; Liu, J., Selection of DNA Aptamers for Sensing Uric Acid in Simulated Tears. *Anal. Sens.* **2022**, DOI: 10.1002/anse.202200010.
- (30) Luo, Y.; Jin, Z.; Wang, J.; Ding, P.; Pei, R., The Isolation of a DNA Aptamer to Develop a Fluorescent Aptasensor for the Thiamethoxam Pesticide. *Analyst* **2021**, *146*, 1986-1995.
- (31) Carlotti, B.; Fuoco, D.; Elisei, F., Fast and Ultrafast Spectroscopic Investigation of Tetracycline Derivatives in Organic and Aqueous Media. *PCCP* **2010**, *12*, 15580-15591.
- (32) Li, Z.-j.; Qi, W.-n.; Feng, Y.; Liu, Y.-w.; Ebrahim, S.; Long, J., Degradation Mechanisms of Oxytetracycline in the Environment. *J. Integr. Agric.* **2019**, *18*, 1953-1960.
- (33) Yang, K.-A.; Pei, R.; Stojanovic, M. N., In Vitro Selection and Amplification Protocols for Isolation of Aptameric Sensors for Small Molecules. *Methods* **2016**, *106*, 58-65.

- (34) Slavkovic, S.; Zhu, Y.; Churcher, Z. R.; Shoara, A. A.; Johnson, A. E.; Johnson, P. E., Thermodynamic Analysis of Cooperative Ligand Binding by the Atp-Binding DNA Aptamer Indicates a Population-Shift Binding Mechanism. *Sci. Rep.* **2020**, 10, 18944.
- (35) Slavkovic, S.; Johnson, P. E., Isothermal Titration Calorimetry Studies of Aptamer-Small Molecule Interactions: Practicalities and Pitfalls. *Aptamers* **2018**, 2, 45-51.
- (36) Liu, X.; Hou, Y.; Chen, S.; Liu, J., Controlling Dopamine Binding by the New Aptamer for a FRET-Based Biosensor. *Biosens. Bioelectron.* **2020**, 112798.
- (37) Lisi, S.; Fiore, E.; Scarano, S.; Pascale, E.; Boehman, Y.; Ducongé, F.; Chierici, S.; Minunni, M.; Peyrin, E.; Ravelet, C., Non-SELEX Isolation of DNA Aptamers for the Homogeneous-Phase Fluorescence Anisotropy Sensing of Tau Proteins. *Anal. Chim. Acta* **2018**, 1038, 173-181.
- (38) Li, Y.; Zhao, Q., Aptamer Structure Switch Fluorescence Anisotropy Assay for Small Molecules Using Streptavidin as an Effective Signal Amplifier Based on Proximity Effect. *Anal. Chem.* **2019**, 91, 7379-7384.
- (39) Yeasmin Khusbu, F.; Zhou, X.; Chen, H.; Ma, C.; Wang, K., Thioflavin T as a Fluorescence Probe for Biosensing Applications. *TrAC, Trends Anal. Chem.* **2018**, 109, 1-18.
- (40) Sun, C.; Su, R.; Bie, J.; Sun, H.; Qiao, S.; Ma, X.; Sun, R.; Zhang, T., Label-Free Fluorescent Sensor Based on Aptamer and Thiazole Orange for the Detection of Tetracycline. *Dyes Pigm.* **2018**, 149, 867-875.
- (41) Gustavsson, T.; Markovitsi, D., Fundamentals of the Intrinsic DNA Fluorescence. *Acc. Chem. Res.* **2021**, 54, 1226-1235.
- (42) Shoara, A. A.; Slavkovic, S.; Donaldson, L. W.; Johnson, P. E., Analysis of the Interaction between the Cocaine-Binding Aptamer and Its Ligands Using Fluorescence Spectroscopy. *Can. J. Chem.* **2017**, 95, 1253-1260.
- (43) Armstrong-Price, D. E.; Deore, P. S.; Manderville, R. A., Intrinsic "Turn-on" Aptasensor Detection of Ochratoxin a Using Energy-Transfer Fluorescence. *J. Agric. Food. Chem.* **2020**, 68, 2249-2255.
- (44) Autour, A.; C. Y. Jeng, S.; D. Cawte, A.; Abdolazadeh, A.; Galli, A.; Panchapakesan, S. S.; Rueda, D.; Ryckelynck, M.; Unrau, P. J., Fluorogenic RNA Mango Aptamers for Imaging Small Non-Coding Rnas in Mammalian Cells. *Nat. Commun.* **2018**, 9, 656.
- (45) Truong, L.; Ferré-D'Amaré, A. R., From Fluorescent Proteins to Fluorogenic RNAs: Tools for Imaging Cellular Macromolecules. *Protein Sci.* **2019**, 28, 1374-1386.
- (46) Kuang, Y.; Jia, H.; Miyanaga, K.; Tanji, Y., Effect of Milk on Antibacterial Activity of Tetracycline against Escherichia Coli and Staphylococcus Aureus Isolated from Bovine Mastitis. *Appl. Microbiol. Biotechnol.* **2009**, 84, 135-142.
- (47) Nutiu, R.; Li, Y., Structure-Switching Signaling Aptamers. *J. Am. Chem. Soc.* **2003**, 125, 4771-4778.

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