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Article

Capture-SELEX for Chloramphenicol Binding Aptamers for Labeled and Label-Free Fluorescence Sensing

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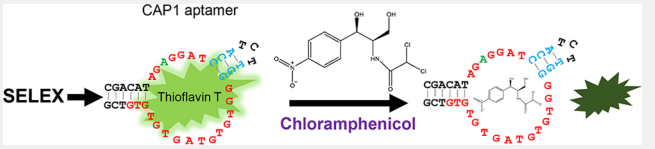
ABSTRACT: Chloramphenicol (CAP) is a potent antibiotic. Due to its side effects, CAP is currently banned in most countries, but it is still found in many food products and in the environment. Developing aptamer-based biosensors for the detection of CAP has interested many researchers. While both RNA and DNA aptamers were previously reported for CAP, they were all obtained by immobilization of the CAP base, which omitted the two chlorine atoms. In this work, DNA aptamers were selected using the library-immobilized method and free unmodified CAP. Three families of aptamers were obtained, and the best one named CAP1 showed a dissociation constant (K_d) of 9.8 μM using isothermal titration calorimetry (ITC). A fluorescent strand-displacement sensor showed a limit of detection (LOD) of 14 μM CAP. Thioflavin T (ThT) staining allowed label-free detection of CAP with a LOD of 1 μM in buffer, 1.8 μM in Lake Ontario water, and 3.6 μM in a wastewater sample. Comparisons were made with previously reported aptamers, and ITC failed to show binding of a previously reported 80-mer aptamer. Due to the small size and well-defined secondary structures of CAP1, this aptamer will find analytical applications for environmental and food monitoring.



KEYWORDS: *aptamers, biosensors, SELEX, chloramphenicol, antibiotics, fluorescence*

INTRODUCTION

Chloramphenicol (CAP) is a broad-spectrum antibiotic used to treat a wide range of bacterial infections by binding to prokaryotic ribosomes and preventing peptide bond formation. Due to its many side effects such as aplastic anemia, leukemia, and gray baby syndrome,[1] its use has been banned in most countries.[2] However, its continued illicit use has led to residues being found in the environment, which can increase antibiotic resistance and contaminate the environment.[3]

Aside from instrumentation-based analytical methods, detection of CAP has usually been achieved with immunoassays.[4,5] For small molecule detection, immunoassays have to be performed in a competitive format. More recently, aptamer-based detection has gained popularity.[6−11] Aptamers are single-stranded oligonucleotides that can selectively bind target molecules. For small molecule targets, aptamers have various "signal-on" detection methods,[10] which is an advantage compared to antibodies. Aptamers are generally found through a process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment).[12−14] RNA aptamers for CAP with dissociation constants (K_d) <100 μM were previously isolated by Burke et al. in 1997.[15] These RNA aptamers were mainly used to study the RNA-antibiotic interactions and not for developing biosensors. In 2011, DNA aptamers for CAP with reported K_d of 0.8−1 μM were reported by Mehta et al., which were obtained by immobilization of the CAP base (Figure 1A).[16] In the CAP base, the two chlorine atoms were removed from the CAP structure, which can also compromise the quality of the obtained aptamers. While the full-length aptamer is 80 nt, a truncated 40 nt sequence has been used for the development of various biosensors.[17−19] However, there were varied reports on the binding affinity of this truncated aptamer to CAP.[17,20] In 2016, Duan et al. used a similar immobilization chemistry and reported another set of aptamers for CAP, where the claimed K_d reached 0.03 to 0.1 μM.[21] However, no systematic truncation was performed, and the full-length aptamers were around 80 nt, which were much less used for the detection of CAP due to its length.[22]

All of the previously mentioned CAP aptamers were isolated by the immobilization of a CAP analogue onto a solid substrate.[15,16,21] This method disallowed access to the entire target due to the covalent linker. In order to access all parts of unmodified target molecules, an alternative to target immobilization was developed called capture-SELEX.[13,23−26] Capture-SELEX involves immobilization of a DNA library onto a substrate and then the target is flowed through to harvest eluted aptamers. Many aptamers with good binding affinities have been selected using this method.[27−30] In this study, we selected a few short DNA aptamers for CAP using the capture-SELEX method and compared our new aptamer with previously reported ones. The use of the new aptamer for

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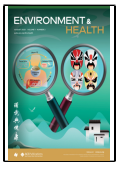

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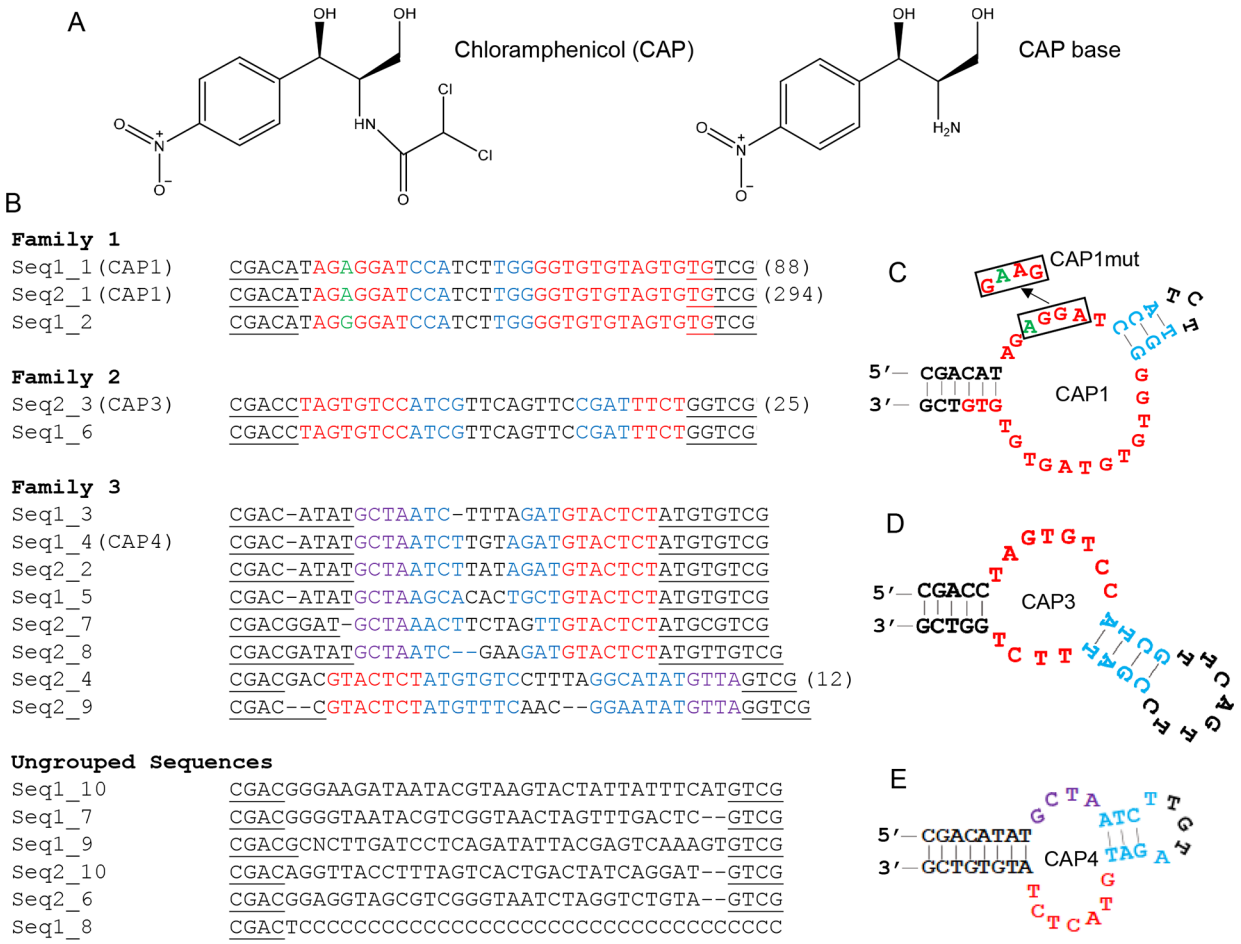

Figure 1. (A) Structures of CAP and CAP base. (B) Top 10 most abundant sequences from the first (Seq1) and the second (Seq2) sequencing. The bases in red are conserved within each family, whereas the remaining sequences have little to no homology. The number of copies of each sequence is in brackets for the selected sequences. The sequences without a copy number contained less than 10 copies. Secondary structures of the (C) CAP1, (D) CAP3, and (E) CAP4 aptamers predicted using Mfold.

the detection of CAP in real water samples was also demonstrated.

MATERIALS AND METHODS

Chemicals

All of the DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). The DNA sequences and modifications are listed in Table S1. CAP and other chemicals were from Sigma-Aldrich. The reagents for aptamer selection were mainly from New England Biolabs.[30] CAP was dissolved in 1× selection buffer (1× SB, 50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$) at final concentrations of 5, 1, and 0.1 mM for the selection. CAP solution was freshly made for each round of the selection to avoid potential degradation.[31] The wastewater was from the City of Kitchener, ON, Canada.

Capture-SELEX for CAP

Our selection was done using the previously described capture-SELEX method.[30] The initial concentration of CAP was 5 mM dissolved in 1× SB. The concentration of CAP was reduced at round 9 to 1 mM and at round 12 to 0.1 mM. A total of 13 rounds of selection was done before the final library was sent for deep sequencing (McMaster University). Since the convergence of the library was low, four more rounds of selection were carried out using the library from the end of the previous round 11 using 1 mM CAP.

Isothermal Titration Calorimetry (ITC)

ITC was performed using a MicroCal VP-ITC. First, 0.4 mM of CAP was titrated into a 20 μM aptamer (CAP1 and CAP3) at 25 °C. For CAP4, 2 mM CAP was titrated into 50 μM aptamer. Aptamers were preannealed at 90 °C for 5 min in 1× SB and allowed to cool to room temperature before loading into the ITC cell. Both the target and the aptamer were dissolved in the same selection buffer. Background heat of titrating antibiotics into the buffer was subtracted. Data were analyzed using the accompanying Origin software.

Biosensor Based on the Strand Displacement Reaction

A carboxyfluorescein (FAM)-labeled aptamer (5 μM) and a quencher-labeled strand (10 μM) were first hybridized in 1× SB by heating for 3 min at 95 °C followed by gradual cooling to room temperature. This hybridized solution was diluted to 100 nM in 1× SB 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 stable background signal. Then various concentrations of CAP were added after the background had stabilized. The same was repeated for real water samples.

Thioflavin-T (ThT)-Based Fluorescence Sensing

The CAP1 aptamer (1 μM) and ThT (500 nM) were mixed in Tris-HCl buffer pH 7.8 with 10 mM Mg^{2+}. The fluorescence emission at 495 nm was first read with an excitation wavelength of 425 nm. The background signal was run for approximately 12 min to ensure a stable background before CAP was added and fluorescence changes

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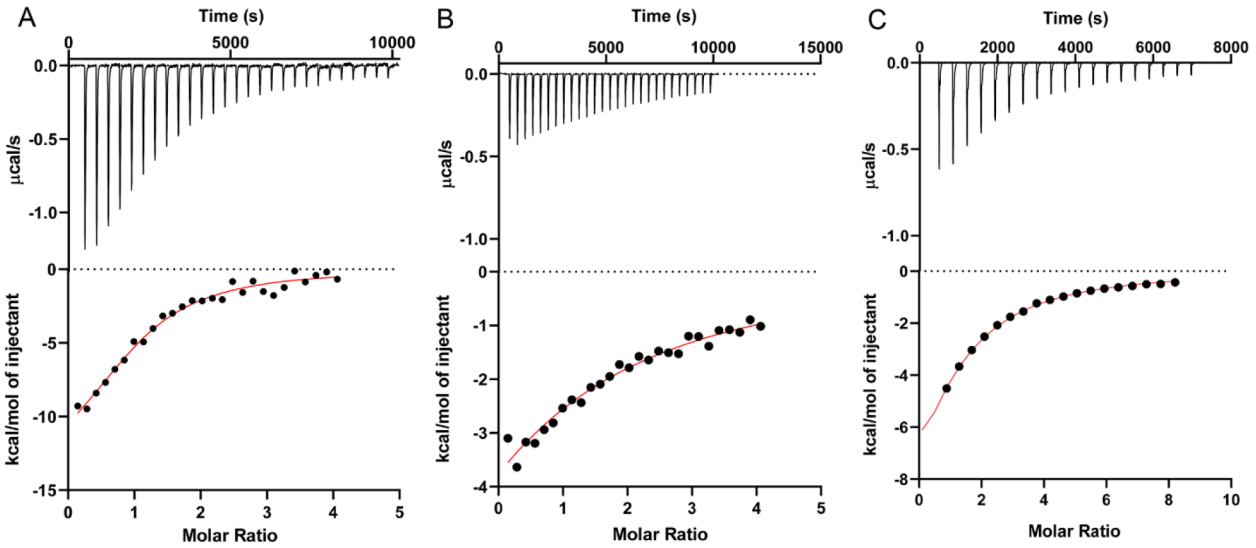

Figure 2. ITC traces of (A) 0.4 mM CAP into 20 μM CAP1 aptamer, (B) 0.4 mM CAP into 20 μM of CAP3, and (C) 2 mM CAP into 50 μM CAP4 at 25 °C. Aptamers were preannealed at 90 °C for 5 min in 1× SB.

were recorded. The buffer was then replaced with real water samples for the detection.

RESULTS AND DISCUSSION

Capture Selection of CAP Binding Aptamers

The structure of CAP is shown in Figure 1A (left). Unlike the previous selections using immobilized CAP base (Figure 1A, right), our capture-SELEX method used unmodified CAP. The DNA library contained a region of 30 random nucleotides flanked on each side by a primer binding region.[30,32] The primer binding regions formed a stem, which would fold the library into a hairpin structure.[13] The library was first hybridized with a short complementary strand bearing a biotin tag. Streptavidin-coated agarose beads were used as the substrate for immobilization. Upon incubation with CAP, aptamer sequences were released from the beads by the formation of the hairpin structure, collected, and amplified using polymerase chain reaction (PCR).

A total of 13 rounds of selection were performed, during which the concentration of CAP was gradually decreased from 5 to 0.1 mM. The round 13 PCR amplicons were deep-sequenced. The sequencing results were processed with the Geneious software, and the most abundant family made up less than 1% of the final library. We suspected that the affinity of CAP binding aptamers might not be very high and using too low of a CAP concentration may lead to relative more nonspecific dissociation. We then decided to keep 1 mM CAP and restarted from the end of round 11 for three more rounds. The final PCR amplicons from the reselected round 14 were also deep-sequenced. A total of 80,865 sequences were obtained, and the sequence with the highest percentage was the same as that in the previous round 13 (before the reselection, Figure 1B).

Three Families of CAP Binding Aptamers

We then aligned the top 10 sequences from both sequencing results together (Figure 1B), and three families were identified. Family 1 contained only three sequences, and we named the most abundant sequence CAP1. The sequences in family 1 appear to be highly conserved and only one position switched from a guanine to an adenine. Two identical sequences were found in family 2, and we named this sequence CAP3 (the third most abundant sequence from the first sequencing result). Family 3 contained a lot more sequences, although each at a lower abundance, and we can identify two conserved regions linked by a small hairpin structure. Circular permutation was observed for these two conserved regions, which is often an indication of the same way of aptamer binding to two orientations of the target molecule (flipped by 180°).[29,33,34] A representative sequence in this family is named CAP4. Aside from these, there are also a few orphan sequences that cannot be assigned to any of the above families. Due to their low abundance, they were not analyzed further. The Mfold predicted secondary structures of the three representative aptamers are shown in Figure 1C–E. They all appeared to have an internal hairpin structure. The sequence of the hairpin is conserved in CAP1 and CAP3, whereas CAP4 has a variable hairpin.

CAP1 is more G-rich (>35%) than the other sequences. We also compared our newly selected aptamer to the previously reported CAP aptamers, but did not find any sequence overlap (Figure S1), indicating that we have selected new aptamers. In addition, the RNA aptamer is featured with two A-rich loops, while our aptamer is rich in guanine.

Binding Assay Using ITC

Characterization of aptamer binding was first performed using ITC. ITC is a reliable label-free technique that measures the heat of aptamer binding.[35] In our experiment, 0.4 mM CAP was titrated into 20 μM of CAP1 aptamer at 25 °C in the 1× SB (Figure 2A). The downward spikes indicate an exothermic binding interaction and a dissociation constant (K_d) of 9.8 μM was fitted. A K_d of 9.8 μM is comparable with the previously reported RNA aptamer (Table 1), for which the best K_d was 2.1 μM.[15] During this experiment, we noticed that annealing the aptamer was important. The CAP1 aptamer was annealed by heating the aptamer in the buffer followed by gradually cooling to room temperature before ITC experiments. This was done to promote the formation of proper secondary structures. Without annealing, we obtained a smaller heat (only 64% of the annealed sample) and the data cannot be properly fitted (binding stoichiometry much smaller than 1), suggesting

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Table 1. Thermodynamic Values of Titrating CAP into Various Aptamers Based on ITC

aptamer	N	K_d (μM)	ΔH (cal/mol) ($\times 10^4$)	ΔS (cal/mol/k)
CAP1	1.0 ± 0.1	9.8 ± 0.5	−1.5 ± 0.2	−27.0
CAP3	1.7 ± 0.5	52 ± 14	−8.9 ± 3.6	−10.5
CAP4	0.7 ± 0.1	112 ± 6	−2.9 ± 0.5	−80.2
CAM80				

a fraction of the aptamers were misfolded before annealing (Figure S2).

To further understand the binding of the aptamer, a truncated version of the CAP1 aptamer was tested, where the smaller stem loop was removed (CAP1short), but no binding was observed (Figure S3A). Thus, this hairpin structure is important. We further tested a mutant (CAP1mut) by changing the four bases in the main loop from AGGA to GAAG (Figure 1C, green bases), for which no sign of binding was observed either (Figure S3B). This indicates that the loop may be directly involved in the binding of CAP. These mutation studies indicated that the binding of the CAP1 aptamer to CAP was specific.

CAP3 also showed some binding but to a lesser degree than CAP1 with a fitted K_d of 78 μM (Figure 3A). CAP4 had even weaker binding, and a K_d of 98 μM was obtained (Figure 3C). For CAP1, the binding stoichiometry was 1:1. It has a large entropy loss upon target binding and thus the binding was mainly driven by enthalpy. Overall, CAP1 was our best aptamer candidate. A K_d of 9.8 μM was quite reasonable for a molecule like CAP containing a single aromatic ring and a few hydrogen bond donors/acceptors. For comparison, the classical adenosine aptamer has a K_d of around 6 μM.[36,37] We reason that reports of K_d values down to 0.03 μM CAP[21] might not be from aptamer binding to CAP but from some other events.[38−41]

Comparison with Previously Reported Aptamers

As mentioned in the Introduction, two DNA aptamers and an RNA aptamer were previously reported. One of the DNA aptamers was extensively used, and the secondary structure of this full-length 80-mer aptamer is shown in Figure 3A.[16] The 40-mer sequence in the random region was used in most sensor work (shown in yellow), where the two fixed primer binding regions were removed (Figure 3B).[20] While all the literature showed the structure depicted in the bottom of Figure 3B, this structure was not predicted by Mfold and we saw a structure on the top panel of Figure 3B. We previously did ITC on the 40-mer truncated sequence but observed no binding.[20] We used 10 μM aptamer for that experiment, which should be sufficient if it has a literature reported K_d of around 1 μM. Ma et al. also performed ITC studies on this 40-mer sequence, and they obtained a K_d value of 237 μM CAP, which was much higher than the previously claimed K_d of around 1 μM.[17] Ma et al. used an aptamer concentration of 22 μM and 3 mM CAP for this experiment with buffer conditions of 20 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM KCl, and 2 mM $CaCl_2$ (experimental conditions from personal communications). We previously made the argument that if the full-length 80-mer can bind, the truncated 40-mer is unlikely to bind due to their very different predicted secondary structures.[20] Since previous ITC studies were performed using the truncated 40-mer, we herein also tested the full-length 80-mer (named CAM80 here). Under the same buffer conditions and preannealing as CAP1 (50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$), no binding was observed (Figure 3D). To further confirm it, we also tried the buffer reported in the original paper (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$) but still no binding was observed (Figure S3C). Therefore, our ITC experiments did not support binding of the 80-mer sequence.

The structure of the RNA aptamer for CAP is shown in Figure 3C.[15] It has very interesting adenine-rich loops, which is very different from our reported aptamers. CAP1 is a shorter

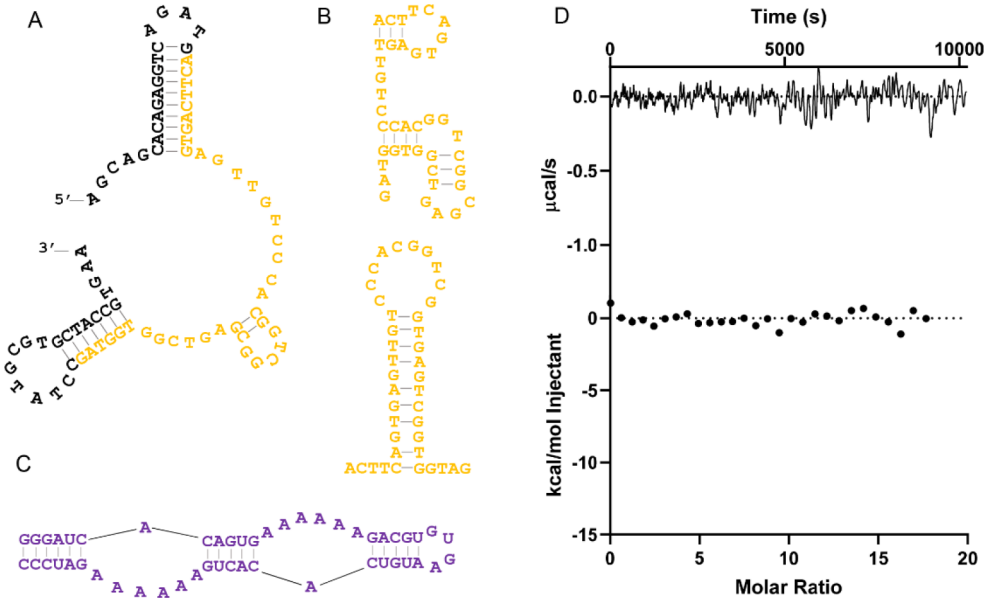

Figure 3. Secondary structures of the (A) full-length 80-mer CAP aptamer and (B) truncated 40-mer of the CAP aptamer (from the yellow region in panel (A)) predicted by Mfold (top) and from the literature (bottom). (C) RNA aptamer for CAP. (D) ITC of 3 mM CAP into 34.5 μM previously reported 80 nt aptamer.

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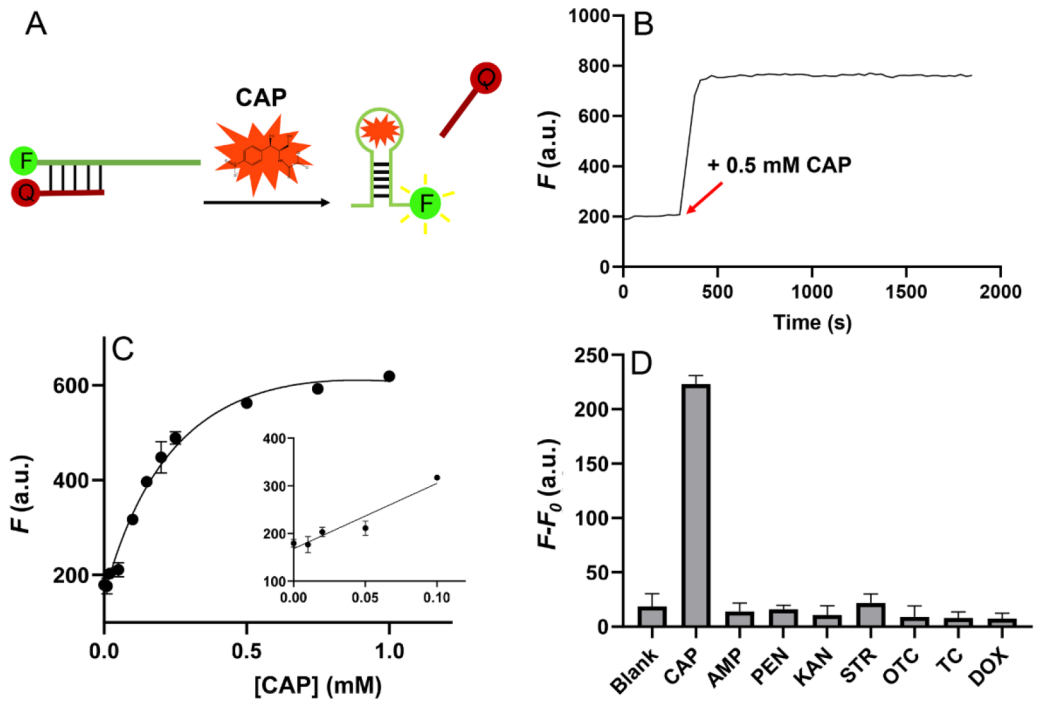

Figure 4. (A) Schematic of the strand displacement fluorescent sensor. (B) Kinetics of 100 nM sensor with the addition of 0.5 mM CAP in 1× SB. (C) Fluorescence intensity as a function of CAP concentration. Inset: linear response at low concentrations. (D) Selectivity of sensor against 0.15 mM antibiotic: penicillin (PEN), kanamycin (KAN), streptomycin (STR), oxytetracycline (OTC), tetracycline (TC), and doxycycline (DOX).

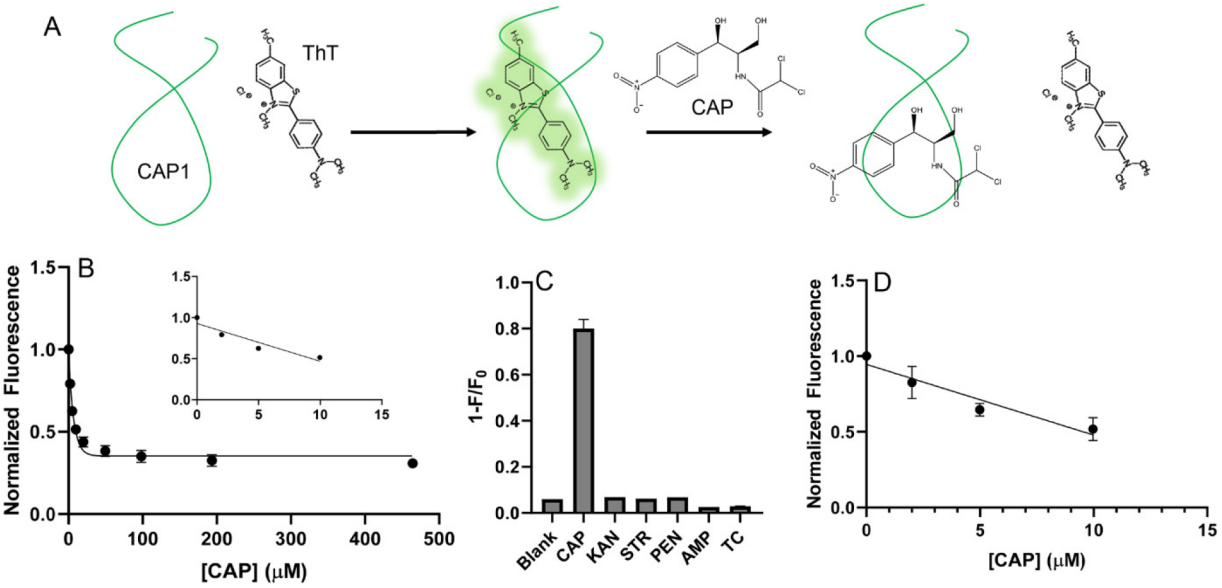

Figure 5. (A) Scheme of using ThT and CAP1 to detect CAP. (B) Calibration curve using 1 μM CAP1 and 0.5 μM ThT in 50 mM, pH 7.8 Tris-HCl buffer with 10 mM $MgCl_2$. Inset: Linear region at low CAP concentrations. (C) Selectivity of the ThT sensor with various antibiotics. (D) Fluorescence intensity after the addition of varying amounts of CAP in filtered Lake Ontario water.

sequence with a well-defined secondary structure. Since DNA has higher stability and lower cost compared to RNA, we believe that CAP1 will be useful for developing analytical biosensors.

Strand Displacement Reaction for Sensing of CAP

After confirming binding, we then designed a strand-displacement fluorescent sensor by using a covalently labeled FAM and quencher system (Figure 4A).[25,42] The CAP1 aptamer was extended on the 5′ end that would be complementary to the 12-mer quencher-labeled strand. The background signal was allowed to run prior to the addition of CAP to ensure a stable background. The fluorescence intensity jumped and stabilized almost instantaneously upon the addition of CAP (Figure 4B). We then varied the concentration of CAP, and the fluorescence was immediately read. A binding curve was obtained with an apparent K_d of 377 μM CAP, much higher than what was measured by ITC. This is due to competition with the complementary strand to increase the apparent K_d value, which was well-documented in the literature.[27,42,43]

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Using this system, a limit of detection of 14 μM CAP was calculated (3σ/slope, where σ is the background fluorescence variation without CAP). A selectivity test was also done using 0.15 mM of other antibiotics (Figure 4D). The sensor showed very good selectivity for CAP as no signal was observed with the other tested antibiotics. This was not surprising since these antibiotics have quite different structures. This limit of detection is not very low, but this assay further confirmed specific aptamer binding. There are many signal amplification methods to drive up the sensitivity.[44−46] Given the similar K_d of CAP1 and the adenosine/ATP aptamer, similar signal amplification methods can also be applied to the detection of CAP.

Label-free Fluorescence Sensing Using ThT

Since the sensitivity of the strand displacement-based assay was low, we further explored a label-free detection method. Thioflavin-T (ThT) is a small molecule that can bind to G-rich DNA, which can enhance its fluorescence intensity.[47] Since the CAP1 aptamer is rich in guanine, we then used ThT staining. By mixing ThT and the CAP1 aptamer, the fluorescence of ThT would increase. CAP binding might displace ThT, leading to decreased fluorescence (Figure 5A). This was previously observed for the adenosine aptamer.[48] The excitation wavelength was set at 425 nm and emission at 495 nm. The CAP1-ThT sensor exhibited a final drop of around 70% when more than 50 μM CAP was added. Using various concentrations of CAP, a calibration curve was generated with a measured K_d of 4.2 μM (Figure 5B). This value was comparable with the value obtained from ITC. In buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$), the limit of detection was calculated to be 1 μM. A high salt concentration is often used in aptamer selection to screen nonspecific electrostatic interactions. For the ThT assay, a lower salt concentration is preferred to increase binding between ThT and DNA. NaCl was determined to be nonessential for CAP1 binding and was thus eliminated from the buffer for the ThT assays (Figure S4). Using this label-free method, we obtained 14-fold better limit of detection compared to using the strand-displacement assay. The binding between ThT and CAP1 aptamer was also studied by titrating up to 10 μM CAP1 into 2.5 μM ThT and a K_d of 2.3 μM was fitted (Figure S5). Given the similar K_d values obtained from ITC and ThT, and the low concentration of ThT used (500 nM), ThT did not serve as a strong competitor in this reaction. The selectivity of this system was then tested against various antibiotics and this system was also very selective for CAP (Figure 5C).

Given the simplicity of this method, we also used ThT staining to test the binding of the CAP3 and CAP4 aptamers. CAP3 showed a K_d of 46 μM (Figure S6), comparable to the value obtained from ITC. The CAP4 data cannot be fitted but the K_d should be close to 100 μM. This experiment also confirmed that CAP1 was the best aptamer for binding CAP.

Real Water Samples with the ThT-Based Sensor

This ThT-based sensing system was then tested using filtered lake water from Lake Ontario. The filtered water was buffered to contain 20 mM Tris-HCl, pH 7.8 with 10 mM $MgCl_2$. The buffered lake water was then spiked with varying concentrations of CAP before 1000 nM CAP1 and 500 nM ThT were added. From this, a linear drop was also observed in low CAP concentrations and a limit of detection of 1.8 μM was obtained (Figure 5D). This value was comparable with that in the clean buffer, confirming that this aptamer can also work in environmental water samples. A similar experiment was conducted using even more challenging a wastewater sample, in which a limit of detection of 3.6 μM was obtained (Figure S7).

CONCLUSIONS

We used the library immobilization method to obtain a few new DNA aptamers that bind the antibiotic, CAP. The best aptamer named CAP1 came from the most abundant family after sequencing and showed a K_d of 9.8 μM using ITC. CAP1 shares no sequence homology with previously claimed aptamers for CAP. ITC was also done with a previously selected 80-nt aptamer, which did not support its binding with CAP. An advantage of the CAP1 aptamer is its short length and well-defined secondary structure showing the structure-switching property (switching from the complementary DNA binding state to the target binding state).[42] A fluorescent strand-displacement sensor showed a limit of detection of 14 μM. The CAP1 aptamer can also enhance the fluorescence of ThT. This fluorescence could then be lowered with the addition of CAP, and a limit of detection of 1 μM was achieved this way. Sensing was also demonstrated in both a lake water sample and a wastewater sample. This newly selected aptamer can be integrated into many sensing platforms for the detection of CAP.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.3c00017.

DNA sequences; sequence alignment with a previously reported aptamer; additional ITC traces for control sequences; additional ThT-based binding assays for other aptamers (PDF)

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Notes

The authors declare no competing financial interest.

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