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Screening and Identification of DNA Aptamers against T-2 Toxin Assisted by Graphene Oxide

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Supporting Information

ABSTRACT: A high-affinity ssDNA aptamer that specifically binds to T-2 toxin was generated by the systemic evolution of ligands by exponential enrichment (SELEX) procedure assisted by graphene oxide (GO). After 10 rounds of selection against T-2 toxin, a highly enriched ssDNA pool was sequenced and the representative aptamers were subjected to binding assays to evaluate their affinity and specificity. Circular dichroism spectroscopy was also used to study the inherent interaction of T-2 toxin and the preferred aptamer Seq.16, which demonstrated a low dissociation constant (K_d) of 20.8 ± 3.1 nM and excellent selectivity for T-2 toxin. Using the selected aptamer Seq.16 as the recognition element, an aptamer-based fluorescent bioassay was developed for the measurement of T-2 in beer samples with a linear range from 0.5 to 37.5 μM (R^2 = 0.988) and a limit of detection (LOD) of 0.4 μM. The results indicate that GO−SELEX technology is appropriate for the screening of aptamers against small-molecule toxins, offering a promising application for aptamer-based biosensors.

KEYWORDS: *T-2 toxin, mycotoxin, aptamer, SELEX, graphene oxide (GO)*

INTRODUCTION

T-2 toxin (T-2), the International Union of Pure and Applied Chemistry (IUPAC) name of which is (2α,3α,4β,8α)-4,15-bis(acetyloxy)-3-hydroxy-12,13-epoxytrichothec-9-en-8-yl-3-methylbutanoate), is one of the most toxic trichothecene mycotoxins produced by various species of *Fusarium*, mainly including *Fusarium acuinatum*, *Fusarium poae*, *Fusarium langsethiae*, and *Fusarium sporotrichioide*.[1−3] The toxin is widely distributed in grains, including maize, oats, barley, wheat, and rice, as well as in some cereal-based products all over the world.[4] As a nonvolatile, low-molecular-weight compound (MW = 466 g/mol), T-2 is resistant to degradation for 6−7 years at room temperature and cannot be inactivated even by autoclaving.[5,6] T-2 has posed a serious risk to humans and animals, leading to emesis, diarrhea, lethargy, weight loss, hemorrhage, immunosuppression, necrosis, cartilage damage, apoptosis, and even death.[7−9] Therefore, T-2 toxin is considered to be one of the most dangerous contaminants by the European Food Safety Authority (EFSA).[9]

Until recently, the estimation of trace amounts of T-2 present in food samples because of fungal contamination has been a difficult task.[10] One type of current analytical technique available for T-2 detection is instrumental analysis, including liquid chromatography−tandem mass spectrometry (LC−MS/MS),[9] ultra-performance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS),[11] and gas chromatography−tandem mass spectrometry (GC−MS/MS).[12] These instrument-based analytical methods rely on expensive, sophisticated equipment and trained personnel and also require laborious and complicated sample preparation processes, including extraction, cleanup, and/or derivatization, and, thus, are not pervasive. Another type of technique includes immunoassays, such as enzyme-linked immunosorbent assays (ELISAs)[8,13] and immunoassay-based rapid strip tests,[14] which offer desired sensitivity and are widely used for simultaneous analysis of a large number of samples. However, precise preparation and proper storage of high-quality anti-T-2 antibodies are essential for immunoassay methods; otherwise, false-positive or negative results may routinely occur because of antibody instability. Because the preparation of antibodies against T-2 haptens is rather tedious and time-consuming, we aim here to develop an alternative recognition molecule to anti-T-2 antibodies. Aptamers can selectively bind to their target molecules with high affinity, rivaling that of antibodies, which are ssDNA or RNA oligonucleotides generated by systemic evolution of ligands by exponential enrichment (SELEX) technology.[15,16] These ligands are relatively easy to obtain, because they are prepared *in vitro* without animal immunization and are more stable against biodegradation.[17] All of these unique properties render aptamers ideal recognition elements for detecting specific target molecules.

However, the screening of aptamers against T-2 is relatively difficult because such small-molecule targets must be immobilized on matrixes, such as sepharose[18,19] or magnetic beads,[20,21] in most SELEX methods. These procedures not only

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involve complex chemical reactions to immobilize the targets and their analogues onto the matrixes but also alter the inherent structure of the target molecules, possibly leading to a reduction in aptamer affinity toward their original targets. Although Yang and Bowser reported that they screened aptamers for *N*-methyl mesoporphyrin IX (MW = 580 g/mol) by capillary electrophoresis (CE)−SELEX, allowing for the binding and separation steps to occur in free solution without steric hindrance,[22] CE−SELEX employs expensive capillary electrophoresis as an instrumental platform for SELEX, which limits its application in aptamer selection. Recently, Park et al. reported an immobilization-free method for screening aptamers against insulin assisted by graphene oxide (GO)[23] as well as against whole bovine viral diarrhea virus type 1 via a similar method.[24] GO is a well-known graphene derivative; one of its important properties is that ssDNA can be strongly adsorbed on its surface because of hydrophobic and $\pi-\pi$ stacking interactions between the nucleobases and GO, whereas dsDNA adsorption on GO is very weak because of the shielding of nucleobases by the phosphate backbone.[25,26]

In the present study, we used GO for screening aptamers against the small-molecule target T-2. Measures were taken during SELEX to generate high-quality aptamers for T-2. The incubation system throughout the selection process contained 1.0% (volume ratio) of methanol to increase the compatibility of T-2 and the oligonucleotides. Because T-2 is relatively insoluble in water,[6] the toxin was dissolved in methanol, a common extraction solvent used in current detection, which enabled T-2 to gain full access to the ssDNAs in free solution. In addition, common mycotoxins that are potentially found in combination with T-2 were added, starting at the sixth round of SELEX to increase the specificity of the generated aptamers for T-2. The presented methodology eliminates the necessity of solid-phase attachment, avoids structural changes in the target molecule, reduces the potential for non-specific interactions, and does not rely on expensive instrumentation. Therefore, our proposed method is believed to be a more versatile technique than previously reported methods to a certain extent.

MATERIALS AND METHODS

Chemicals and Reagents. The initial ssDNA library and primers for amplification were synthesized by Integrated DNA Technologies (Coralville, IA). T-2, fumonisin B_1 (FB_1), and ochratoxin A (OTA) were obtained from Fermentek, Ltd. (Jerusalem, Israel); zearalenone (ZEN), aflatoxin B_1 (AFB_1), and acrylamide/bis-acrylamide (30% solution) were purchased from Sigma-Aldrich (St. Louis, MO). The operators were required to wear gloves and masks to avoid exposure to the above toxins. GO was prepared from graphite powder using a modified Hummer's method,[27] as described previously by our group.[28] A homogeneous suspension of GO (approximately 5.0 mg/mL) was prepared by ultrasonic vibration and used throughout the experiment. The reagents for polymerase chain reaction (PCR) amplification and 5′-carboxyfluorescein (FAM)-labeled aptamers were obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). Lambda exonuclease enzyme (5000 units/mL) and 10× lambda exonuclease reaction buffer were purchased from New England BioLabs (Ipswich, MA). Chromatographically pure methanol and other unspecified chemicals and reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Solutions were prepared with ultrapure water processed with a Millipore Direct-Q3 ultrapure water system (Billerica, MA).

Apparatus. All PCRs were performed in a C1000 thermocycler (Hercules, CA). The observation of polyacrylamide gel was carried out using a Chemi Doc XRS+ imaging system with Image Lab software (Hercules, CA). A ND-1000 spectrophotometer (Wilmington, DE) was used to measure the nucleic acid concentration. Fluorescent detection was performed using a F-7000 fluorescence spectrophotometer (Tokyo, Japan). Circular dichroism (CD) spectroscopy was performed on a MOS-450/AF-CD (Claix, France) spectropolarimeter interfaced with a computer with Biokine V4.51 software installed.

Generation of ssDNA Library. An 80 nucleotide oligonucleotide ssDNA library consisting of a central 40 nucleotide randomized region flanked by two 20 nucleotide primer binding sites was used as the initial library (5′-CAGCTCAGAAGCTTGATCCT-N40-GACTC-GAAGTCGTGCATCTG-3′) on the basis of the study by Tang et al.[29] The ssDNA library and subsequent aptamer pools were amplified by symmetric PCR with a forward primer (5′-CAGCTCAGAAGCTT-GATCCT-3′) and phosphorylated reverse primer (5′-P-CAGATG-CACGACTTCGAGTC-3′) under the following conditions: 5 min at 94 °C, repeated cycles of 30 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. The PCR cycles were optimized in each round of SELEX to obtain the intended 80 base pair products. The PCR products were analyzed using 8% native PAGE and purified by a PCR Product Purification Kit (Generay Biotech. Co., Ltd., Shanghai, China). Subsequently, the concentrations of recovered products were measured using a ND-1000 spectrophotometer to estimate the amount of lambda exonuclease needed. The phosphorylated antisense strands were digested by lambda exonuclease according to the instructions of the supplier (New England BioLabs). After identification using 8% denaturing PAGE with 7 M urea, the digestion products were purified, precipitated, and subsequently used as the ssDNA library.

***In Vitro* Selection.** The GO−SELEX process is illustrated in Scheme 1. Prior to being mixed with target mycotoxin T-2, the ssDNA library dissolved in binding buffer (BB; 10 mM Tris-HCl, 150 mM NaCl, 10 mM KCl, and 2.5 mM $MgCl_2$ at pH 7.4)[30] was heated at 94 °C for 5 min, immediately cooled on ice for 15 min, and placed at 25 °C for 10 min. In the first round, folded ssDNA (1 nmol) was incubated with T-2 (100 nmol) in 400 μL of special binding buffer, which contained 1.0% methanol, for 2 h at 25 °C with tilting and rotation. Subsequently, 10.0 mg of GO (mass ratio of GO/ssDNA is 400:1) from the above-mentioned GO suspension by centrifugation were added to the mixture of potential aptamers and unbound ssDNA, and the solution was incubated at 25 °C for 40 min. This mixture containing GO was centrifuged at 13 000 rpm/min for 10 min, and the supernatant containing ssDNAs bound to T-2 was collected, while the deposit with unbound ssDNAs adsorbed onto GO was discarded. The collected potential aptamer pool was amplified by PCR, and the ssDNA products were generated by lambda exonuclease digestion of the phosphorylated strands from dsDNA products. The subsequent round of GO−SELEX was performed with the purified library.

Counter GO−SELEX was performed starting from the sixth round to improve the specificity of the aptamers against T-2. The ssDNA library (200 pmol) was first incubated with a mixture of FB_1, ZEN, AFB_1, or OTA (5 nmol, individually) in 400 μL of incubation buffer for 60 min, and then GO (2.0 mg) was added and incubated for 40 min. During this process, the oligonucleotides that did not bind to the counter-targets were adsorbed onto the GO surface by $\pi-\pi$ stacking

Scheme 1. Schematic Illustration of the Aptamer Selection Procedure

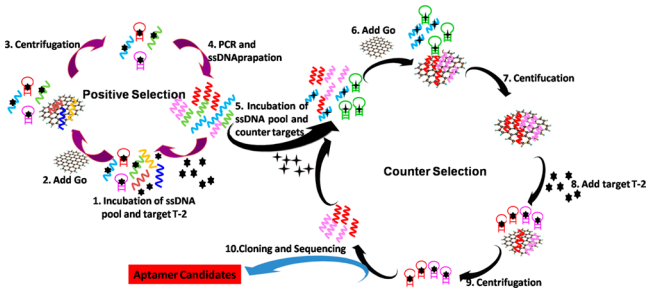

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Table 1. Total of 27 Sequences (Primers Omitted) Obtained after Cloning and Sequencing[a]

family	code number	variable N_{40} sequences from the 21st to 60th position (5′−3′)	nucleotide	ΔG	occurrence
	Seq.1	TATTCGTCAAGTCCTCTGCCACATTACGCTCTTAACGCTG	40	−7.0	1
F1	Seq.2	TAGCACACAACCCTTTGATTGGGATATCTCATCCCTCG	38	−11.5	20
	Seq.19	GTCTTAGATTAGATTAAAAAACTTATATTGCTAAAACCCG	40	−5.7	1
F2	Seq.6	ACCACAGCCAGTAAGTCCCGTAGGCCCTGGTTGGGCTGTT	40	−14.5	1
	Seq.9	ATGTCATATTACACTACTCATTCGTGCTTTCTTCGTTTT	39	−9.9	1
F3	Seq.12	GTAGCTTAAAATTTATGGAGGTATGTTTCACTTTTCATTT	40	−4.9	1
	Seq.16	GTATATCAAGCATCGCGTGTTTACACATGCGAGAGGTGAA	40	−17.3	2

[a]The affinity and K_d of the underlined sequences were characterized using binding assays.

interactions, while those bound to the counter-targets remained suspended in the buffer. After separation by centrifugation, GO on which oligonucleotides were adsorbed was resuspended and washed with BB 5 times for harsher conditions. Subsequently, target T-2 (20 nmol) was mixed with the GO−ssDNAs and incubated further for 2 h in 400 μL of buffer, to recover the aptamers from the GO surface. The mixture solution was centrifuged, and the supernatant containing potential aptamers was collected. This obtained library pool was amplified by PCR, and ssDNA was again generated by lambda exonuclease digestion and purification.

Cloning and Sequencing of the Enriched Pool. Following 10 rounds of selection, the enriched ssDNA pool was amplified by unmodified primers. The purified PCR product was cloned using the pMD-19T cloning vector (Takara Biotech.Co., Ltd., Dalian, China) and transformed into *Escherichia coli* DH5α. Through blue−white spot screening, 30 white positive clones were randomly selected and cultured in LB medium containing 50 μg/mL ampicillin, followed by plasmid DNA extraction using a Plasmid MiniPrep Kit (Generay Biotech. Co., Ltd., Shanghai, China). Finally, the extracted plasmid DNA with inserted aptamer candidates were sequenced by Sangon Biotech. Co., Ltd. (Shanghai, China). Multiple sequence alignment of the obtained sequences was analyzed using DNAMAN software,[31] and the secondary structure of each sequence was predicted using RNAstructure 4.6 software (http://rna.urmc.rochester.edu/RNAstructure.html) with paraments of maximum percent energy difference of 10 and maximum number of structures of 20.

Binding Assays. The aptamers were heat-denatured and cooled on ice, using the same procedure as for the *in vitro* selection, prior to use. To identify individual aptamer candidates, binding assays for each FAM-labeled representative sequence and its truncated sequence (without the primer-binding regions) were conducted by incubating a fixed amount of T-2 (1 μM) with various concentrations of the synthetic sequences, ranging from 5 to 200 nM, in 500 μL of BB in the dark for 2 h. Then, GO proportional to the amount of ssDNA present was added to each tube, and the mixture was incubated in the dark for 40 min. After centrifugation, the fluorescence intensity of the T-2/aptamer complexes in the supernatant was measured using a F-7000 fluorescence spectrophotometer with excitation at 490 nm. Negative controls without T-2 added were used to determine non-specific binding. All of the binding assay experiments were repeated 3 times. The equilibrium dissociation constant (K_d) of aptamer/T-2 binding was determined using GraphPad Prism 5.0 software based on a nonlinear fit of the T-2/aptamer complex fluorescence intensities for a range of aptamer concentrations.

Evaluation of Specificity. Three sequences with lower K_d values were selected to evaluate the specificity of the aptamers against T-2 based on the binding assay results. The specificity test was performed as follows: GO (0.3 mg/mL) in the special binding buffer was incubated with the aptamers (100 nM), along with T-2 or FB_1, ZEN, AFB_1, or OTA (10 μM, individually), for 2 h in the dark. The negative control for each sequence was mixed with BB instead of any mycotoxin targets. Subsequently, each of the mixtures was centrifuged at 13 000 revolutions per minute (rpm) for 10 min, and the supernatant was collected and kept in the dark. Finally, the fluorescence of each supernatant was measured using a F-7000 fluorescence spectrophotometer with excitation at 490 nm.

CD Spectroscopy Assay. To further investigate the mechanism of binding between the preferred aptamer Seq.16 and target T-2, CD spectroscopy was used to study the conformation of the ssDNA aptamer before and after binding with free T-2 in free solution. Aptamer Seq.16 (1 μM) was incubated with or without target T-2 (20 μM) for 2 h in special binding buffer as well as a tube of pure T-2 (20 μM) in the vibration oven (total volume of each was 400 μL). Subsequently, these samples were analyzed on a MOS-450/AF-CD (France) spectropolarimeter with recording of the CD spectra from 230 to 320 nm.

Aptamer-Based Fluorescent Bioassay. The potential application of aptamer Seq.16 for the detection of T-2 in beer was explored by a fluorescent bioassay. Canned beer purchased from a local supermarket (50 mL) was completely degassed by sonication, and then the CO_2-free beer was filtered through a 13 mm, 0.22 μm nylon syringe filter. Prior to being used in the study, the beer sample was analyzed by mass spectrometry to make sure that T-2 was undetectable. The beer was spiked with the stock solution of T-2 to obtain a final concentration of 1 mM T-2. From this spiked sample, several dilutions from 0.05 to 500 μM T-2 were prepared with non-spiked beer. Then, 400 μL of folded 5′-FAM-labeled Seq.16 (125 nM) in 5× BB was mixed with 100 μL of beer sample at a final concentration of T-2 ranging from 0.01 to 100.0 μM, along with GO (150 μg). The control group was set similarly but without T-2 added. The mixtures (500 μL) were incubated in the dark for 2 h with gentle shaking. Subsequently, each of the mixtures was centrifuged at 14 000 rpm for 10 min, and the fluorescent intensity of the supernatant collected was measured using a F-7000 fluorescence spectrophotometer with excitation at 490 nm.

RESULTS AND DISCUSSION

Screening of Aptamers against T-2. Several preliminary experiments were conducted before the screening of aptamers against T-2. The critical point at which approximately 500 μg of GO could adsorb 100 pmol of the ssDNA pool was first determined by an adsorption test using the initial library pool. The double ratio of GO/ssDNA was maintained throughout the selection process to ensure that the ssDNAs not bound to T-2 were completely adsorbed. Next, a series of PCRs containing 0, 0.1, 1.0, or 10.0% (volume ratio) methanol in the PCR system was performed to study the effect of the methanol content in the special binding buffer on PCR amplification (see the Supporting Information). Finally, 1.0% methanol was selected for use in the incubation system during *in vitro* selection, enabling the T-2 toxin full access to ssDNAs in free solution. A balance was achieved between control of the selection conditions and PCR amplification, which greatly contributed to the screening of aptamers against T-2.

During the formal SELEX process, T-2 was used as the target, and several common mycotoxins, including FB_1, ZEN, AFB_1, and OTA, were treated as counter-targets. The counter-targets are known to be produced by the same fungus species or to likely coexist with the target mycotoxin in a heterogeneous environment. Five rounds of counter selection following five

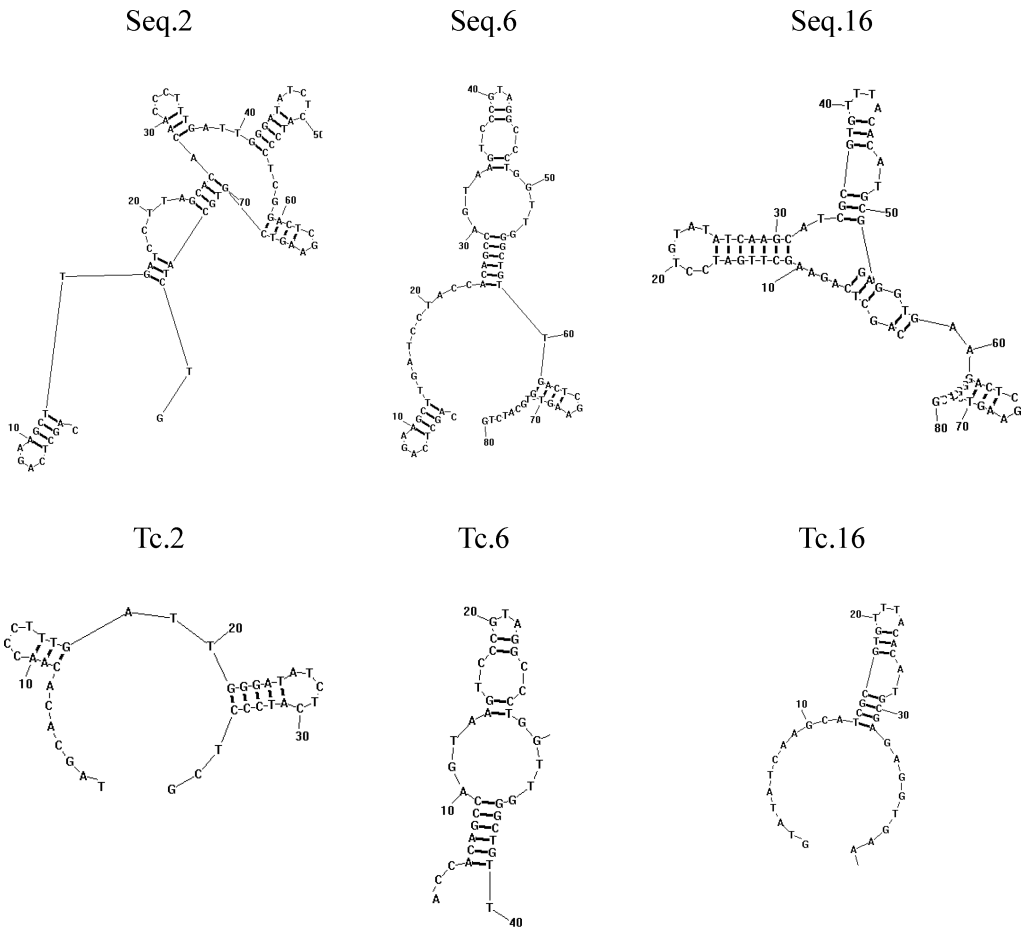

Figure 1. Secondary structure models of representative aptamers and their truncated sequences predicted by software RNAstructure 4.6.

rounds of positive selection increased the specificity of aptamers by the efficient removal of non-specific sequences in each round. The recovery amount of ssDNA bound to T-2 was measured by a ND-1000 spectrophotometer, and selection was finished in the 10th round when we observed that the recovery ratio of it was much higher than that of the first round. The above-mentioned GO−SELEX procedure eliminated the necessity for solid-phase attachment, thus avoiding structural changes in the target molecule, reducing the potential for non-specific interactions, and not relying on expensive instrumentation.

Preliminary Identification of Aptamers. After the 10th round of immobilization-free SELEX, a total 30 recombinant plasmids were sent for sequencing. Finally, 27 sequences of aptamer candidates were successfully obtained from Sangon Biotech. Co., Ltd., and they were grouped into three families, as presented in Table 1. The aptamer candidate termed Seq.2 was the sequence with the highest abundance in the enriched library, but two bases were missing in the variable N40 region. As Viswanathan et al. reported, GC-rich regions in the templates can cause the formation of stem-loop secondary structures that have been known to promote polymerase jumping during PCR amplification.[32] This polymerase jumping produces smaller PCR products with missing sequences for the template stem-loop region.[33] Similar cases have been reported for the study by McKeague et al. of fumonisin B_1 aptamers[34] and the study by Kiani et al. of digoxin aptamers.[35] However, these missing bases would not matter if Seq.2 exhibited high affinity and specificity toward target T-2.

Representative aptamers from each family were selected on the basis of DNA homology, as analyzed using DNAMAN software, and the lowest predicted free energy of formation ΔG, as calculated using RNAstructure 4.6 software. The sequences that occurred twice or more in the cloning results were chosen because these sequences may be present in higher abundance. Meanwhile, aptamers with lower ΔG were also chosen, because these sequences should possess more stable secondary structures, which help to maintain the stability of the target/aptamer complexes. The secondary structures of the representative aptamers Seq.2, Seq.6, and Seq.16 and their truncated sequences Tc.2, Tc.6, and Tc.16 predicted by RNAstructure 4.6 software are shown in Figure 1. Unique stems, loops, bulges, hairpins, triplexes, and pseudo-knots assembled in the aptamers may have contributed to the specific binding of these aptamers to target T-2.

Affinity of DNA Aptamers to T-2. The binding saturation curves of the measured fluorescence intensity of the T-2/aptamer complexes (after deduction of the blank background) plotted against the aptamer concentration (5, 10, 25, 50, 100, or 200 nM) were generated, and the K_d values for the aptamer sequences were calculated by nonlinear regression analysis using GraphPad Prism 5.0 (Figure 2). Among the tested sequences, the K_d values of Seq.2 (62.4 ± 12.1 nM), Seq.6 (37.2 ± 8.4 nM), and Seq.16 (20.8 ± 3.1 nM) were relatively lower than that of Tc.6 (81.3 ± 21.7 nM), indicating higher affinities to T-2. The binding of the aptamers to their targets typically resulted from structure compatibility, stacking of aromatic rings, electrostatic interactions, van der Waals forces,

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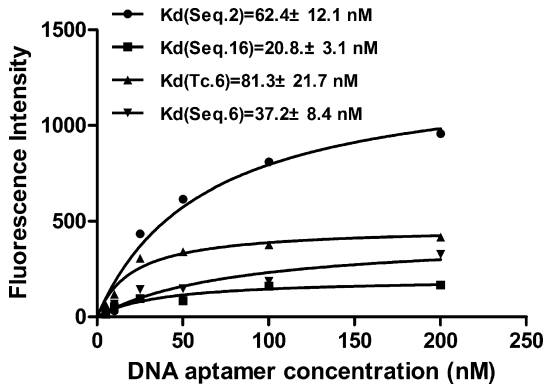

Figure 2. Binding saturation curves and determination of the dissociation constants (K_d) of aptamers against T-2 by nonlinear regression analysis using GraphPad Prism 5.0.

and hydrogen-bond interactions or a combination of these effects.[36] As seen in Figure 1, the secondary structure of the short sequence Tc.6 is similar to that of Seq.6 but the secondary structures of Tc.2 and Tc.16 remarkably differed from those of their full-length sequences. This difference might be the reason for the lack of affinity of sequence Tc.2 and Tc.16 to target T-2, which was most likely caused by the truncation of primer-binding regions that resulted in a failure to form the three-dimensional structures essential for binding.

Specificity of Aptamers. On the basis of the results of the affinity assays, three aptamers (Seq.2, Seq.6, and Seq.16) with lower K_d values for T-2 were selected for specificity testing. The specificity tests were performed using a competitive binding method inspired by the report by Lu et al.[37] GO was mixed with each FAM-labeled aptamer, along with the target molecule T-2 or with FB_1, ZEN, AFB_1, or OTA. In the presence of T-2, the binding between the FAM-labeled DNA and target molecules should alter the conformation of the FAM-labeled DNA and disturb the interaction between the ssDNA and GO, thus shifting the FAM-labeled DNA into the supernatant.[37] From Figure 3, all of the three aptamer candidates (Seq.2, Seq.6, and Seq.16) exhibited obviously higher corrected

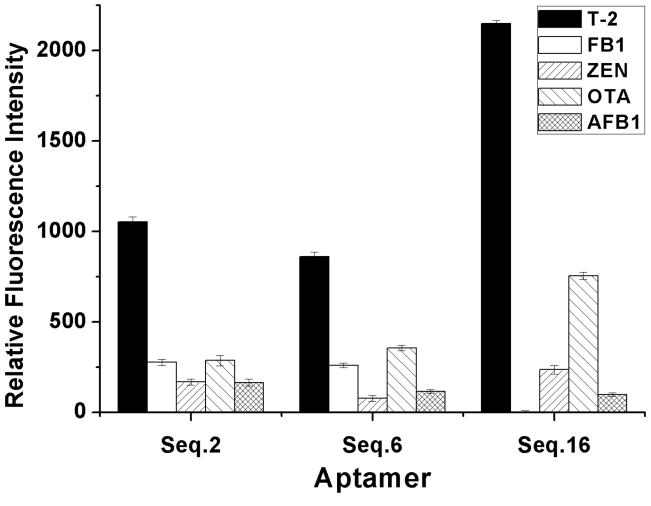

Figure 3. Characterization of the specificity of aptamers Seq.2, Seq.6, and Seq.16 by a competitive binding assay. The data shown are corrected values with the negative control signal value deducted. Each data point represents the mean ± standard deviation of three replicates.

fluorescence intensities in the presence of T-2, in comparison to incubation with the counter-targets, and aptamer Seq.16 was found to be significantly more specific to target T-2.

Study of the Interaction Mode between T-2 and Aptamer. The CD spectroscopy assay is a powerful and sensitive technology to study the interaction mode between small molecules and DNA aptamers. For further study of conformational information on Seq.16, CD spectra were recorded before and after binding with T-2 in special binding solution. As shown in Figure 4, the CD spectrum of pure T-2 is

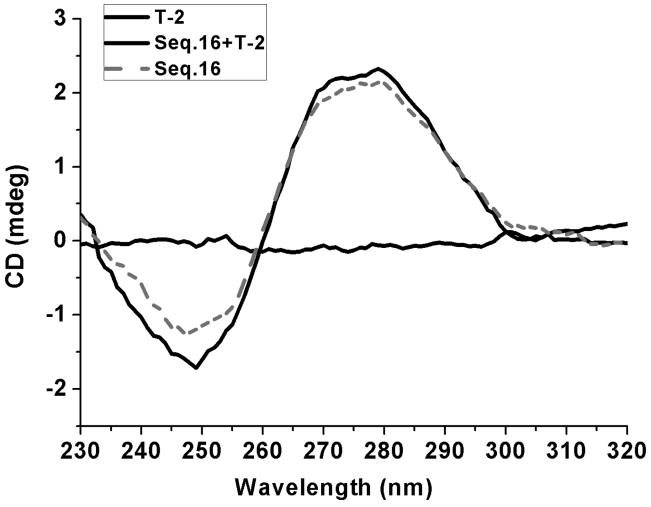

Figure 4. CD spectra of T-2 and aptamer Seq.16 in the absence or presence of T-2. The final concentration of T-2 is 20 μM, and that of the aptamer is 1 μM.

near the baseline, almost zero signal. The CD spectrum of pure aptamer Seq.16 displays a negative band at 249 nm and a positive band at 280 nm, indicating that the aptamer may have an A-form duplex of the hairpin structure.[38] The addition of T-2 caused a slight increase of the positive band around 280 nm and an apparent decrease of the negative band around 250 nm, suggesting that the binding of T-2 to Seq.16 could promote the formation of the A-form duplex of the hairpin structure.

Application of Aptamer Seq.16. To demonstrate the potential application of aptamer Seq.16, we performed a fluorescent bioassay for the detection of T-2 in beer samples. Matrix background interference from beer was reduced by diluting the sample 5 times with BB. Because of the high affinity and specificity of Seq.16 against the target T-2, the binding events between the aptamers and targets disturb the interaction between the ssDNA and GO, thus reserving the FAM-labeled aptamers in the supernatant. In the absence of T-2, the fluorescent intensity of the diluted beer sample was at a minimum; in the presence of different concentrations of T-2, the fluorescent intensity varied. A calibration curve of aptamer-based detection of T-2 in beer was created by plotting the relative fluorescence intensity (ΔI) against the T-2 concentration of undiluted beer (Figure 5), in which ΔI represents the difference of fluorescence intensity in the presence and absence of T-2. A linear correlation (R^2 = 0.988) was obtained between 0.5 and 37.5 μM (0.1 and 7.5 μM in the diluted beer sample). The sensitivity of the developed bioasssay was investigated, and the limit of detection (LOD) for T-2 was found to be 0.4 μM in the real beer sample. Recovery tests were performed for evaluation of the accuracy of the developed method for T-2

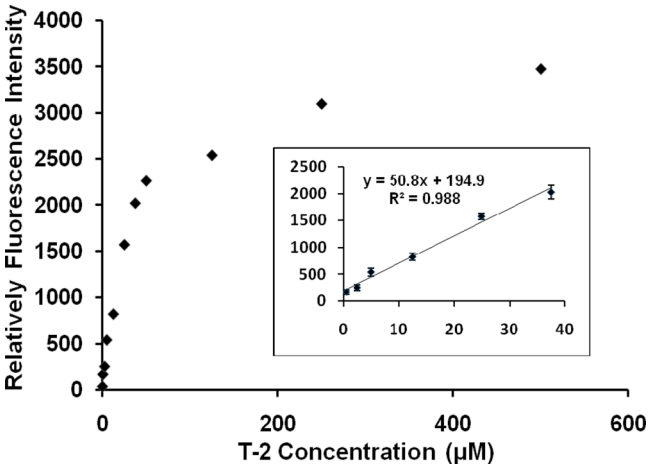

Figure 5. Calibration curve for T-2 in beer measured by the aptamer-based fluorescent bioassay.

determination in beer. A good assay recovery for spiked real beer samples was obtained, as listed in Table 2. The preliminary study in the work shows potential application of the selected aptamer in the detection of T-2 in real samples. A further study is ongoing in our laboratory to develop advanced biosensors for T-2 detection, with an increased sensitivity and a decreased detection limit, by means of the signal amplification technique.

Table 2. Recovery of T-2 in Spiked Beer by the Aptamer-Based Fluorescent Bioassay

T-2 added (μM)	T-2 detected (μM) (n = 3)	recovery (%)
2.5	2.34 ± 0.14	93.6
12.5	12.6 ± 0.12	100.8
25.0	26.5 ± 0.12	106.0

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Impact of methanol on PCR amplification. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Li, Y.; Wang, Z.; Beier, R.; Shen, J.; De Smet, D.; De Saeger, S.; Zhang, S. T-2 toxin, a trichothecene mycotoxin: Review of toxicity, metabolism, and analytical methods. *J. Agric. Food Chem.* **2011**, *59*, 3441−3453.

(2) Pascale, M.; Panzarini, G.; Visconti, A. Determination of HT-2 and T-2 toxins in oats and wheat by ultra-performance liquid chromatography with photodiode array detection. *Talanta* **2012**, *89*, 231−236.

(3) Sibanda, L.; De Saeger, S.; Peteghem, C.; Grabarkiewicz-Szczesna, J.; Tomczak, M. Detection of T-2 toxin in different cereals by flow-through enzyme immunoassay with a simultaneous internal reference. *J. Agric. Food Chem.* **2000**, *48*, 5864−5867.

(4) European Food Safety Authority (EFSA). *Occurrence Data of Trichothecene Mycotoxins T-2 Toxin and HT-2 Toxin in Food and Feed*; EFSA: Parma, Italy, 2010; http://www.efsa.europa.eu/en/supporting/pub/66e.htm (accessed May 31, 2011).

(5) Wannemacher, R. W.; Wiener, S. L. Trichothecene mycotoxins. In *Textbook of Military Medicine*; Bellamy, R. F., Ed.; Borden Institute, Office of The Surgeon General, Department of the Army: Washington, D.C., 1997, Chapter 34, pp 655−676.

(6) Duffy, M. J.; Reid, R. S. Measurement of the stability of T-2 toxin in aqueous solution. *Chem. Res. Toxicol.* **1993**, *6*, 524−529.

(7) Raisbeck, M. F.; Rottinghaus, G. E.; Kendall, J. D. In *Effects of Naturally Occurring Mycotoxins on Ruminants*; Smith, J. E., Henderson, R. S., Eds.; CRC Press: Boca Raton, FL, 1991; Vol. *241*, pp 647−677.

(8) Li, Y.; Luo, X.; Yang, S.; Cao, X.; Wang, Z.; Shi, W.; Zhang, S. High specific monoclonal antibody production and development of an ELISA method for monitoring T-2 toxin in rice. *J. Agric. Food Chem.* **2014**, *62*, 1492−1497.

(9) Sun, Y.; Zhang, Y.; Zhao, H.; Zheng, J.; Hu, F.; Fang, B. Liquid chromatography−tandem mass spectrometry method for toxicokinetics, tissue distribution, and excretion studies of T-2 toxin and its major metabolites in pigs. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2014**, *958*, 75−82.

(10) Pal, A.; Acharya, D.; Saha, D.; Dhar, D. K. Development of a membrane-based immunofiltration assay for the detection of T-2 toxin. *Anal. Chem.* **2004**, *76*, 4237−4240.

(11) Soleimany, F.; Jinap, S.; Faridah, A.; Khatib, A. A UPLC−MS/MS for simultaneous determination of aflatoxins, ochratoxin A, zearalenone, DON, fumonisins, T-2 toxin and HT-2 toxin, in cereals. *Food Control* **2012**, *25*, 647−653.

(12) Kong, W.; Zhang, X.; Shen, H.; Yang, Z.; Yang, M. Validation of a gas chromatography−electron capture detection of T-2 and HT-2 toxins in Chinese herbal medicines and related products after immunoaffinity column clean-up and pre-column derivatization. *Food Chem.* **2012**, *132*, 574−581.

(13) Barna-vetro, I.; Gyongyosi, A.; Solti, L. Monoclonal antibody-based enzyme-linked immunosorbent assay of fusarium T-2 and zearalenone toxins in cereals. *Appl. Environ. Microbiol.* **1994**, *60*, 729−731.

(14) Molinelli, A.; Grossalbe, K.; Führer, M.; Baumgartner, S.; Sulyok, M.; Krska, R. Development of qualitative and semiquantitative immunoassay-based rapid strip tests for the detection of T-2 toxin in wheat and oat. *J. Agric. Food Chem.* **2008**, *8*, 2589−2594.

(15) Ellington, A. D.; Szostak, J. W. In vitro selection of RNA molecules that bind specific ligands. *Nature* **1990**, *346*, 818−822.

(16) Tuerk, C.; Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **1990**, *249*, 505−510.

(17) Niazi, J. H.; Lee, S. J.; Gu, M. B. Single-stranded DNA aptamers specific for antibiotics tetracyclines. *Bioorg. Med. Chem.* **2008**, *16*, 7245−7253.

(18) Cruz-Aguado, J. A.; Penner, G. Determination of ochratoxin A with a DNA aptamer. *J. Agric. Food Chem.* **2008**, *56*, 10456−10461.

(19) Song, K. M.; Cho, M.; Jo, H.; Min, K.; Jeon, S. H.; Kim, T.; Han, M. S.; Ku, J. K.; Ban, C. Gold nanoparticle-based colorimetric detection of kanamycin using a DNA aptamer. *Anal. Biochem.* **2011**, *415*, 175−181.

(20) Mehta, J.; Van Dorst, B.; Rouah-Martin, E.; Herrebout, H.; Scippod, M. L. In vitro selection and characterization of DNA

aptamers recognizing chloramphenicol. *J. Biotechnol.* **2011**, *155*, 361−369.
(21) Song, K. M.; Jeong, E.; Jeon, W.; Jo, H.; Ban, C. A coordination polymer nanobelt (CPNB)-based aptasensor for sulfadimethoxine. *Biosens. Bioelectron.* **2012**, *33*, 113−119.
(22) Yang, J.; Bowser, M. T. Capillary electrophoresis−SELEX selection of catalytic DNA aptamers for a small-molecule porphyrin target. *Anal. Chem.* **2013**, *85*, 1525−1530.
(23) Park, J. W.; Tatavarty, R.; Kim, D. W.; Jung, H. T.; Gu, M. B. Immobilization-free screening of aptamers assisted by graphene oxide. *Chem. Commun.* **2012**, *48*, 2071−2073.
(24) Park, J. W.; Lee, S. J.; Cho, E. J.; Kim, J.; Song, J. Y.; Gu, M. B. An ultra-sensitive detection of a whole virus using dual aptamers developed by immobilization-free screening. *Biosens. Bioelectron.* **2014**, *51*, 324−329.
(25) Wu, M.; Kempaiah, R.; Huang, P.; Maheshwari, V.; Liu, J. Adsorption and desorption of DNA on graphene oxide studied by fluorescently labeled oligonucleotides. *Langmuir.* **2011**, *27*, 2731−2738.
(26) Chen, C.; Zhao, J.; Jiang, J.; Yu, R. A novel exonuclease III-aided amplification assay for lysozyme based on graphene oxide platform. *Talanta* **2012**, *101*, 357−361.
(27) Hummers, W. S.; Offeman, R. E. Preparation of graphitic oxide. *J. Am. Chem. Soc.* **1958**, *80*, 1339.
(28) Wu, S.; Duan, N.; Ma, X.; Xia, Y.; Wang, H.; Wang, Z.; Zhang, Q. Multiplexed fluorescence resonance energy transfer aptasensor between upconversion nanoparticles and graphene oxide for the simultaneous determination of mycotoxins. *Anal. Chem.* **2012**, *84*, 6263−6270.
(29) Tang, J.; Yu, T.; Guo, L.; Xie, J.; Shao, N.; He, Z. *In vitro* selection of DNA aptamer against abrin toxin and aptamer-based abrin direct detection. *Biosens. Bioelectron.* **2007**, *22*, 2456−2463.
(30) Qi, C.; Bing, T.; Mei, H.; Yang, X.; Liu, X.; Shangguan, D. G-quadruplex DNA aptamers for zeatin recognizing. *Biosens. Bioelectron.* **2013**, *41*, 157−162.
(31) Duan, N.; Ding, X.; He, L.; Wu, S.; Wei, Y.; Wang, Z. Selection, identification and application of a DNA aptamer against *Listeria monocytogenes*. *Food Control* **2013**, *33*, 239−243.
(32) Viswanathan, V. K.; Krcmarik, K.; Cianciotto, N. P. Template secondary structure promotes polymerase jumping during PCR amplification. *BioTechniques* **1999**, *27*, 508−11.
(33) Kang, J.; Lee, M. S.; Gorenstein, D. G. The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: Application to in vitro combinatorial selection of aptamers. *J. Biochem. Biophys. Methods* **2005**, *64*, 147−151.
(34) McKeague, M.; Bradley, C. R.; Girolamo, A. D.; Visconti, A.; Miller, J. D.; DeRosa, M. C. Screening and initial binding assessment of fumonisin B1 aptamers. *J. Mol. Sci.* **2010**, *11*, 4864−4881.
(35) Kiani, Z.; Shafiei, M.; Rahimi-Moghaddama, P.; Karkhaneb, A. A.; Ebrahimi, S. A. In vitro selection and characterization of deoxyribonucleic acid aptamers for digoxin. *Anal. Chim. Acta* **2012**, *748*, 67−72.
(36) Ruff, K. M.; Snyder, T. M.; Liu, D. R. Enhanced functional potential of nucleic acid aptamer libraries patterned to increase secondary structure. *J. Am. Chem. Soc.* **2010**, *132*, 9453−9464.
(37) Lu, C. H.; Yang, H. H.; Zhu, C. L.; Chen, X.; Chen, G. N. A graphene platform for sensing biomolecules. *Angew. Chem., Int. Ed.* **2009**, *48*, 4785−4787.
(38) Bozza, M.; Sheardy, R. D.; Dilone, E.; Scypinski, S.; Galazka, M. Characterization of the secondary structure and stability of an RNA aptamer that binds vascular endothelial growth factor. *Biochemistry* **2006**, *45*, 7639−7643.