JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Isolation and Identification of the DNA Aptamer Target to Acetamiprid

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ABSTRACT: As an alternative to antibodies, aptamers have a great potential as analytical tools for pesticide detection. In this work, aptamers targeting acetamiprid were selected by a specific systematic evolution of ligands by exponential enrichment (SELEX) strategy, where a single-stranded DNA (ssDNA) library was immobilized and target modification was eliminated. After 18 rounds of repeated selection, the ssDNA pool was enriched and then 14 sequences were selected and carefully identified. At last, an acetamiprid-specific aptamer with the apparent dissociation constant (K_d) estimated to be 4.98 μM was successfully obtained. Further work is ongoing to develop an aptamer-based detection method for field determination of this pesticides in agricultural products and environmental samples.

KEYWORDS: Aptamer, pesticide, acetamiprid, SELEX

1. INTRODUCTION

Pesticide residue is one of the prominent issues in food safety and environmental pollution, and a rapid pesticide residue detection method is urgently needed to keep humans from being affected. The antibody-based immunoassay (IA) technology is such an analysis system, with simple, rapid, and cost-effective characteristics, and has been used in pesticide detection for a long time. However, the difficulties inherent in antibody generation for small molecules that cannot elicit an immune response and the instability of antibodies under unfavorable environment conditions greatly prevent the wide application of this method.[1] Aptamers are single-stranded DNA (ssDNA) or RNA that can specifically bind to various targets, including small molecules. As an alternative to antibodies, aptamers have a great potential as analytical tools for small molecule detection. Up to now, numerous aptamers with small-molecule-binding specificity have been reported,[2−5] and various aptamer-based biosensor systems (aptasensors) have been developed for small molecule analysis;[6] however, the potential for applying aptamers to pesticide detection has not been given enough consideration. This may be partly due to the process of aptamer selection, which is still complicated to the pesticide analysts.

Since its first description in 1990, the systematic evolution of ligands by exponential enrichment (SELEX) technology[7,8] became the widely applied *in vitro* selection method to evolve aptamers. Briefly, the starting point of each SELEX process is a synthetic random DNA oligonucleotide library consisting of a multitude of ssDNA fragments (or RNA library) with different sequences ($\sim 10^{15}$). The SELEX procedure is characterized by the repetition of successive steps consisting of selection (binding, partition, and elution), amplification, and relevant ssDNA separation. In general, 6−20 SELEX rounds are needed for the selection of high-affinity aptamers. Then, the enriched aptamer pool is cloned, and several individual aptamers have to be characterized.[9] The most crucial aspect of a SELEX process with outstanding importance for the selection of aptamers with high affinity and specificity is the efficient partitioning between target-binding and nonbinding oligonucleotides.[10] For small molecule aptamer selection, target immobilization is traditionally applied to realize the partition process. In this case, the target was immobilized on sepharose[11] or magnetic beads[12] first and then incubated with the oligonucleotide library for binding. However, most of the small molecule targets, especially pesticide targets, cannot directly immobilize on the matrix, and complicated chemical modification is needed to introduce an active group.

In the work by Nutiu and Li,[13,14] they designed a specific DNA library with the goal to directly select structure-switching signaling aptamers. Briefly, this library contained a specific sequence of 15 nucleotides, flanked by two random sequences of 10 and 20 nucleotides, which were further flanked by primer-binding sequences. The specific sequence within the random region was complementary to a biotinylated capture oligonucleotide, and thus, the library could be immobilized on an avidin-coated surface by DNA hybridization. Oligonucleotides that are able to bind to the target should be released from the surface as a result of switching their structures for the formation of the binding complexes. This method eliminates the need for target immobilization for the creation of an affinity column. Hence, although the aim of this study was to create signaling aptamers, it is also very attractive for selection of an aptamer to small molecule targets.

The purpose of this study was to apply the above-mentioned attractive SELEX strategy to select an aptamer target to pesticide molecules. An acetamiprid is one of the widely used insecticides, and we exemplified it by this pesticide.

Received: October 28, 2010
Accepted: January 21, 2011
Revised: January 18, 2011

dx.doi.org/10.1021/jf104189g | *J. Agric. Food Chem.* XXXX, XXX, 000–000

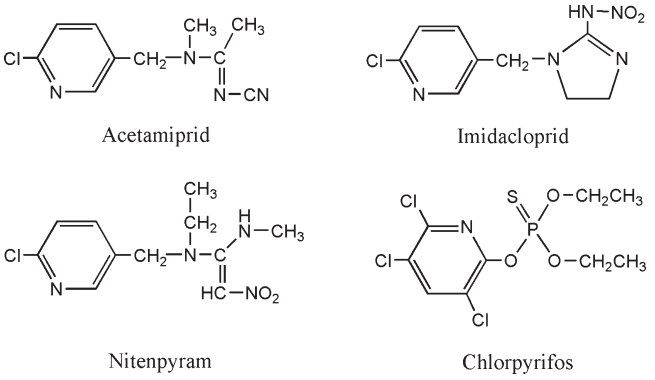

Figure 1. Structure of acetamiprid and three other pesticides used for specificity analysis.

2. MATERIALS AND METHODS

2.1. Chemicals and Apparatus. The acetamiprid (Figure 1) as a target molecule and three other pesticides used for specificity analysis were all purchased from Dr. Ehrensorfer GmbH (Augsburg, Germany). Other unspecified chemicals were analytical-purity. All DNA sequences in Table 1 were chemically synthesized and polyacrylamide gel electrophoresis (PAGE)-purified by Invitrogen (Shanghai, China). The pH of all buffer solutions was measured by Sartorius basic pH-meter PB-10 (Goettingen, Germany). Thermo Pierce streptavidin agarose resins (Rockford, IL) were used for ssDNA library immobilization and relevant ssDNA separation. The fluorescence intensities were recorded on Mithras microplate reader LB 940 (Berthold, Germany) using a black, Corning, Inc. Costar 96-well flat bottom assay plate (catalog number 3915, New York), and excitation and emission were set at 485 and 535 nm, respectively. The equilibrium-filtration analyses of selected aptamers were realized on Millipore YM 10 Microcon filtration columns (Bedford, MA). The concentrations of acetamiprid were analyzed using an Agilent 1200 series high-performance liquid chromatography (HPLC) system with ultraviolet (UV) detector.

2.2. SELEX Procedure. The SELEX procedure applied in this work was based on the method by Nutiu and Li,[13,14] but with some modifications. To avoid the operation of the radioelement in the method by Nutiu and Li, the library was labeled with FAM and the selection was monitored by calculating the ratio of the fluorescence intensity found in the eluted solution and that in the solution of the library before immobilization to streptavidin-coated agarose. In addition, polymerase chain reaction (PCR) amplifications were realized with a biotin-5′-labeled primer P-2 (P-2-B) and a FAM-5′-labeled primer P-3 (F-P-3). The PCR products were incorporated with streptavidin agarose resins for 30 min at room temperature, and then the FAM-labeled sense strand was released by the addition of NaOH and precipitated by ethanol (with glycogen as a carrier) for the next round of selection.[15] After 18 round selections, selected ssDNAs were amplified with a nonmodified primer set and cloned into a pUC-T simple vector using a pUC-T simple cloning kit (CoWin, Beijing, China). Then, white colonies were PCR-identified and followed by the colonies with aptamer inserts sequenced (Invitrogen, Shanghai, China).

2.3. Aptamer Identification. After the SELEX procedure, the selected nucleotides were first analyzed for they ability to signal the presence of acetamiprid as per the method by Nutiu and Li.[13,14] Briefly, the FAM-5′-labeled P-1 sequence (F-P-1), selected nucleotides, DABCYL-3′-labeled BDNA (B-B), and the nonmodified P-2 sequence were dissolved in 1× selection buffer with a ratio of 1:2:8:3 (125, 250, 1000, and 375 nM, respectively), and then the same volume of buffer or acetamiprid solution (final concentration of 0.5 μM) was added. The fluorescence intensities of the buffer- and acetamiprid-added solutions were recorded, and the difference between these two values was defined as the ability of the selected nucleotides to signal the presence of the acetamiprid.

Table 1. DNA Sequences Used in This Work

sequence ID	sequence (5′ → 3′)
library	CCTGCCACGCTCCGCAAGCTT-N10-CTGCAGCGATTCTTGATCG-N20-TAAGCTTGGCACCCGCATCGT (5′-FAM)
P-1	GCGGAGCGTGGCAGG
P-2	ACGATGCGGGTGCCAAGCTTA
P-3	CCTGCCACGCTCCGC
F-P-1	GCGGAGCGTGGCAGG (5′-FAM)
P-2-B	ACGATGCGGGTGCCAAGCTTA (5′-biotin)
F-P-3	CCTGCCACGCTCCGC (5′-FAM)
B-B	TACCGCAAAAAAAAACAAGAATCGCTGCAG (5′-biotin)
Q-B	TACCGCAAAAAAAAACAAGAATCGCTGCAG (3′-DABCYL)

The representative positive nucleotides in the above identification were further identified by the equilibrium-filtration method as per the work by Kim et al.[16] Briefly, selected ssDNA samples (5 μM) were added to acetamiprid in 400 μL of selection buffer at a final concentration of 10 μM. Each mixture was incubated for 30 min at room temperature and then poured into a filtration column and centrifuged for 20 min at 12000*g* to separate the unbound acetamiprid. To compensate for unspecific sorption of acetamiprid, an equivalent concentration of acetamiprid solution without selected ssDNA was passed through the same procedure and then the difference between the normal sample and the ssDNA blank sample was estimated as the amount of acetamiprid−aptamer complexes. The concentration of acetamiprid was analyzed by HPLC under the following conditions: 70:30 acetonitrile/water as the mobile phase, 6 min of running time at a 0.7 mL/min flow rate using the ZORBAX SB-C18 column (5 μm, 4.6 × 255 mm), sample size of 10 μL, and UV detection wavelength of 245 nm.

2.4. Specificity and Affinity Determination. The specificity of the best positive nucleotide was analyzed by comparing its ability to signal the presence of acetamiprid and three commonly used pesticides (imidacloprid, nitenpyram, and chlorpyrifos) as per the method mentioned above.

The apparent dissociation constant (K_d), which is commonly used to describe the affinity, of the best positive nucleotide was further analyzed by the above-mentioned equilibrium-filtration method. In this assay, the ssDNA concentration was keep at 3 μM but the acetamiprid concentration was set as 0.5, 2.5, 5.0, 10.0, 20.0, and 30.0 μM. After equilibrium-filtration analysis, the concentration of the aptamer/acetamiprid complex versus the added chemical concentration was plotted and then the dissociation constant was calculated as per the method reported by Wang et al.[17] Briefly, the result was converted to the concentration of complex (B) versus the binding ratio (B/F, where F is the concentration of free acetamiprid) format and then fitting the result to the following formula:

$$B/F = -1/K_d \cdot B + B_0/K_d$$

2.5. Aptamer Structure Analysis. The secondary structure of selected aptamer was analyzed by the Internet tool MFOLD (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi).

3. RESULTS AND DISCUSSION

3.1. Isolation of Acetamiprid-Specific DNA Aptamers. DNA aptamers binding to acetamiprid were isolated from an

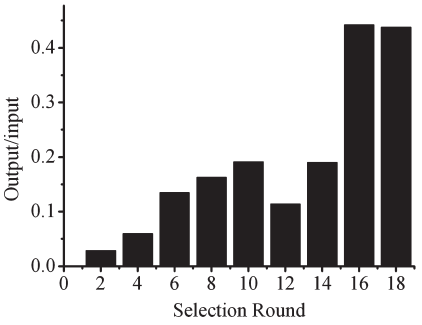

Figure 2. Efficiency of each selection round.

Table 2. Sequence Analysis of Selected ssDNA

aptamer ID	sequences from N10 to N20 (5′ → 3′)
S1	GGTATTAAGTCTGCAGCGATTCTTGATC-GCATTGACTTATTCATGCTTC
S2	TACCTAGAGTCTGCAGCGATTCTTGATC-GTTCTGCAACGCTCTGTACGC
S4	CTTCACTTTTCTGCAGCGATTCTTGATC-GCTAAATGAATGAAGATGTGT
S6	AGTAATAATTCTGCAGCGATTCTTAATC-GCTTGGCACAATATTACTTCC
S7	ATGTGCTATGCTGCAGCGATTCATGATC-GCATCCGCATCTAGTACATCT
S8	TTATAAATTGCTGCAGCGATTCTTGATCG-CGTAATGTTTATGAAAGTTA
S10	ATTGGACTAGCTGCAGCGATTCTTAATCG-CTATGCTCCGCCCAATACTG
S11/S12	AACTAGAGTACTGCAGCGATTCTTGATCG-CTTGGTACCAGTGCTTAGAA
S13	AGTATATTCACTGCAGCGATTATTGATCG-TCAACTGCCATGTGAATAGC
S14	AAATTACACATTGCAGCGATTCTTGATCGT-TAGATGGGTAAAGTTGCTT
S15	TGGTATCCTGCTGCAGCGATTCTTGATCG-CTTCATTGGATACTTATAGA
S18	TGTAATTTGTCTGCAGCGGTTCTTGATCG-CTGACACCATATTATGAAGA
S19	AATCTTACCACTGCAGCGATTCTTAATCG-CTCCCGTGTCGTAAGTACTA
S20	GTAAGTGAGTCTGCAGCGATTCTTGATCG-CTGTTGTGACTTACTCACAC

immobilized random ssDNA library consisting of $10^{14}-10^{15}$ nucleotides (2 nmol). For each selection step, approximately 1 mM acetamiprid were incubated with the immobilized ssDNA library to elute the specific DNA sequences. In the first round of selection, about 1.2% of the initial ssDNA pool was selected. The percentage of ssDNA specific to the target was significantly increased with increasing the SELEX round (Figure 2). In the 16th round of selection, ssDNA showing specificity to acetamiprid was dominated in the DNA pool with 45% of elution yield, and a further two rounds of selection cannot increase the ratio of output/input. Hence, the eluted ssDNAs from the 18th round selection were amplified with a nonmodified primer set and cloned into pUC-T simple vector using pUC-T simple cloning kit, and then 20 white colonies were picked for identification. After PCR identification, the 15 postitive colonies with aptamer inserts were sequenced. In the result of sequencing (Table 2), 2 nucleotides had an indentical sequence; therefore, 14 nucleotide sequences finally selected were further examined in the following experiments.

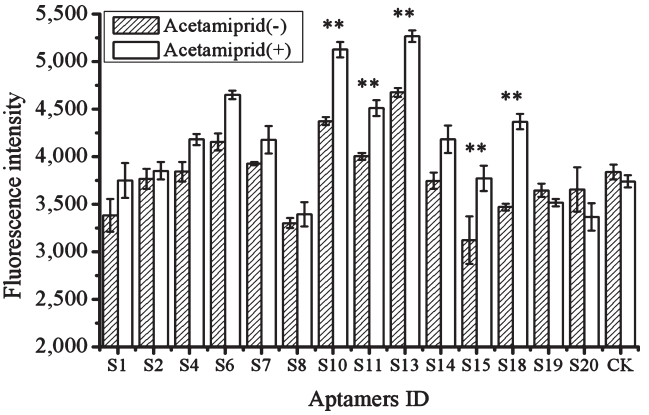

Figure 3. Ability of selected aptamers to signal the presence of acetamiprid. ** indicates that the increase of fluorescence intensities between acetamiprid(+) and acetamiprid(−) is extremely significant ($p < 0.01$).

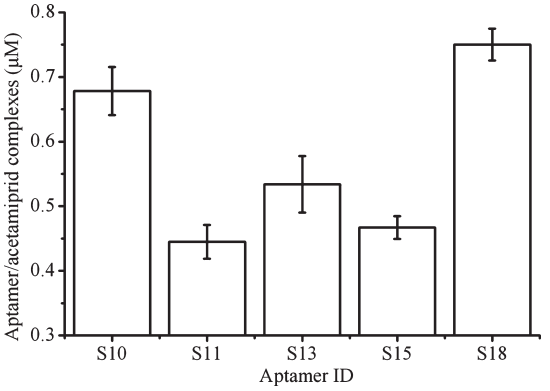

Figure 4. Ability of selected aptamers to form aptamer/acetamiprid complexes.

3.2. Identification of Selected Aptamers. The 14 selected nucleotides were first analyzed for they ability to signal the presence of acetamiprid as per the method by Nutiu and Li. After the target solution was added, an increase of fluorescence intensities was displayed in most of the aptamer identification systems (Figure 3). Alternatively, a decrease of fluorescence intensities was displayed in S19, S20, and CK, and this phenomenon may be the result from the systematic error. Aptamers S10, S11, S13, S15, and S18 were the most active five sequences, and significance analysis showed that the increase of fluorescence intensities in these aptamers was extremely significant ($p < 0.01$). Therefore, they were selected and further identified by the equilibrium-filtration method as per the work by Kim et al. The result indicted that aptamer S18 showed the strongest ability to capture acetamiprid (Figure 4). When 5 μM of this ssDNA was incubated with 10 μM target, 0.75 μM aptamer/acetamiprid complexes can be formed. Hence, the S18 was selected for further study.

3.3. Determination of Specificity and Affinity. The selectivity of the S18 sequence was analyzed by comparing its ability to signal the presence of acetamiprid and three commonly used pesticides (imidacloprid, nitenpyram, and chlorpyrifos). The results (Figure 5a) indicated that S18 selectively signaled the presence of acetamiprid, while the addition of the other three pesticides cannot significantly change the fluorescence intensities in the detection system.

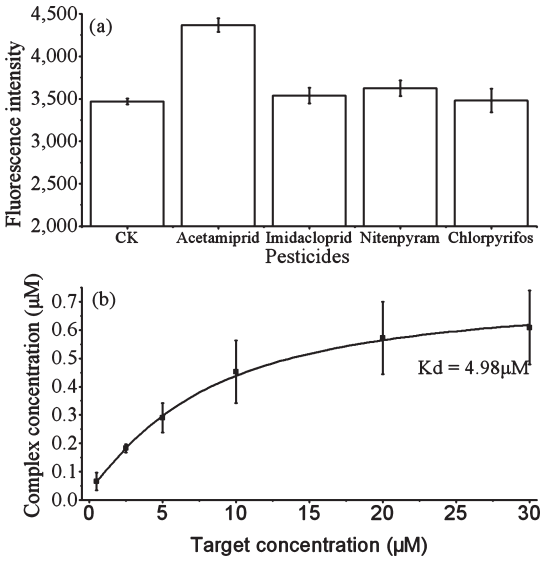

Figure 5. Specificity and affinity determination for S18. (a) Specificity of S18 was analyzed by comparing its ability to signal the presence of different pesticides, and (b) affinity of S18 was detected by the binding assay.

The apparent dissociation constant (K_d) of the S18 sequence was further analyzed. Here, 3 μM of this sequence was incubated with different concentrations of target, and then the concentration of the aptamer/acetamiprid complex was detected by HPLC. The complex concentration versus the added target concentration was plotted in Figure 5b, and it showed that the concentration of complex was increased along with the concentration of acetamiprid. After the result was converted into B versus B/F format, the apparent dissociation constant (K_d) of S18 was estimated to be 4.98 μM and this value was comparable to the K_d value of some published aptamers.[3−5,12]

3.4. Prediction of the Aptamer Structure. The structure of most selected aptamers is characterized by stems, loops, bulges, hairpins, triplexes, or quadruplexes. In addition, the binding of aptamers to their targets typically resulted from structure compatibility, stacking of aromatic rings, electrostatic, van der Waals, and hydrogen bond interactions, or a combination of these effects.[9] To predict the aptamer/acetamiprid-complex-forming process, the secondary structure of the most active five aptamers was analyzed by the Internet tool MFOLD and the results were displayed in Figure 6. In general, the predicted structures of S10

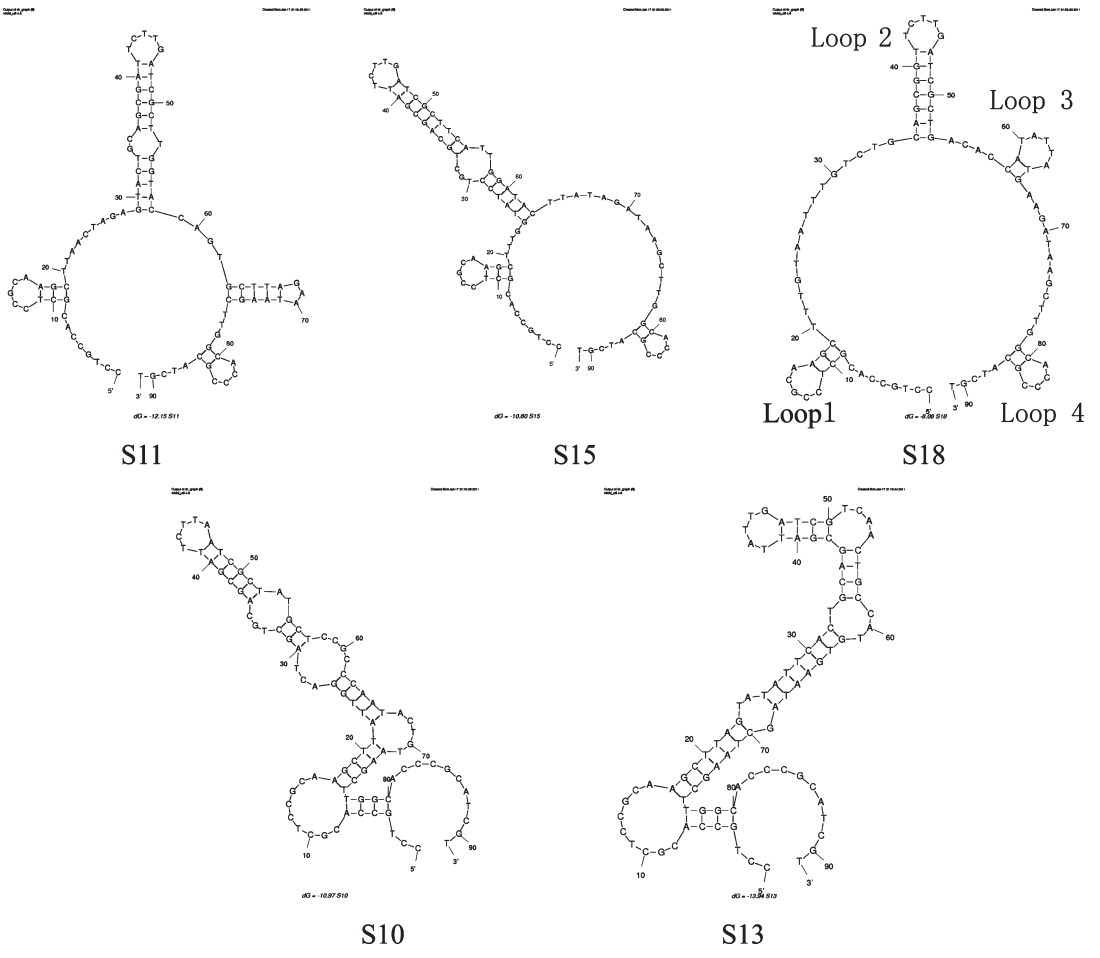

Figure 6. Predicted secondary structures of aptamers S10, S11, S13, S15, and S18.

dx.doi.org/10.1021/jf104189g |J. Agric. Food Chem. XXXX, XXX, 000–000

and S13 are distinctly different from S11, S15, and S18. With regard to the most active aptamer S18, there are 4 loops displayed in its predicted secondary structure, but loops 1, 2, and 4 were formed by the constant region. Hence, the A- and T-rich loop 3, which formed by the random sequences, was predicted to be the target-binding region of S18.

The most crucial aspect of a SELEX process is the efficient partitioning between target-binding and nonbinding oligonucleotides.[10] In most of the published SELEX work, target immobilization is applied to realize this partition process. In this paper, we do this work by immobilizing the ssDNA library to a solid matrix and using a free target to elute the specific ssDNA. Although an acetamiprid-specific aptamer with K_d estimated to be 4.98 μM was successfully obtained here, an aptamer specific to another pesticide target, such as methamidophos, was not generated by this strategy in our group's other work. This may partly be caused by the choice of the library, which was originally designed by Nutiu and Li[13,14] for nucleotide aptamer selection. Parameters such as the size of the library, the arrangement of the random region (for example, here, N10 + N20, but N15 + N15 may be more suitable), and the stability of the library immobilized to the solid phase (it mainly decided by the length of the complementary region between the specific sequence within the random region and the biotinylated capture oligonucleotide) may significantly affect the results of aptamer selection. Hence, rational design of the library is critical to selection aptamer by this method.

In comparison to other published work,[12] a large diversity of the identified nucleotide sequences is obtained here (14 different nucleotide sequences in 15 identified sequences), which indicated that the ssDNA pool can be further enriched. However, after the 16th round selection, the selection yield cannot further improve by the present method. The possible reason of this point is that an unspecific elute was taking place in the negative selection (elute by buffer) and actual selection. This kind of unspecific elute means that dissociation of the complementary region between the ssDNA library and the B-B sequence was taking place without the effect of the target; hence, the efficiency of separation target-binding and nonbinding ssDNA was decreased. We speculate that there is a dynamic balance process of combined dissociation between two cDNA sequences, and the environmental factors, such as pH and ionic strength, can significantly influence this process. Therefore, optimization of the selection condition was also very important for this aptamer selection strategy.

The ultimate aim of our aptamer selection work is developing an aptemer-based pesticide detection method. Although the affinity of our selected aptamer here is lower than typical antibodies, we can couple it with a sophisticated and elegant detection system, such as electrochemical sensors, to fulfill the requirement of detection.

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Funding Sources

The authors gratefully acknowledge the financial support of the National Natural Science Foundation of China (30871658) and the Agriculture Science and Technology Innovation Foundation of Jiangsu Province [CX (10) 236].

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