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Short communication

Organophosphorus pesticides detection using broad-specific single-stranded DNA based fluorescence polarization aptamer assay

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ABSTRACT

An approach is developed to detect the organophosphorus pesticides via competitive binding to a recombinant broad-specificity DNA aptamer with a molecular beacon (MB), the binding of the MB to the aptamer results in the activation of a fluorescent signal, which can be measured for pesticide quantification. Aptamers selected via the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) were structurally modified and truncated to narrow down the binding region of the target, which indicated that loops of the aptamer contributed different functions for different chemical recognition. Thereafter, a variant fused by two different minimum functional structures, was clarified with broad specificity and increased affinity. Further molecular docking and molecular dynamics simulations was conducted to understand the molecular interaction between DNA structure and chemicals. 3D modeling revealed a hot spot area formed by 3 binding sites, forces including hydrogen bonds and van der Waals interactions appear to play a significant role in enabling and stabilizing the binding of chemicals. Finally, an engineered aptamer based approach for the detection of organophosphorus pesticides was successfully applied in a test using a real sample, the limit of quantification (LOQ) for phorate, profenofos, isocarbophos, and omethoate reached 19.2, 13.4, 17.2, and 23.4 nM (0.005 mg L^{-1}), respectively.

1. Introduction

The concern related to pesticide residues in the diet with regard to possible health risks is widespread and has increased in recent years, particularly related to highly poisonous organophosphorus pesticides, such as phorate, profenofos, isocarbophos, and omethoate. Any possible residues of or contamination by these pesticides could have serious consequences for human health. Therefore, the need for a high-throughput screening approach with a predetermined selectivity and affinity for target analytes is greater than ever.

As bio-elements used for recognition, oligonucleotides aptamers exhibit many advantages over other bio-elements, such as their small size, allowing easy synthesis, easy-to-modify, low immunogenic, and chemically stability, and are regarded as promising substitutes for antigen-antibody reactions. Molecular beacon (MB) is a nucleic acid motif with a hairpin-shaped structure that has one fluorophore and one non-fluorescent quencher covalently linked to each end of its stem, resulting in low fluorescence, and the loop sequence is complementary to the target sequence as a probe (Tyagi and Kramer, 1996; Yamamoto et al., 2000). When the MB encounters a target molecule, the binding of the target disrupts the stem and separates the fluorophore from the quencher, leading to a fluorescence signal. Tuerk and Gold (1990) accomplished the selection of T4 DNA polymerase-binding sequences from an RNA pool and referred to this selection procedure as Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Moreover, with the development of SELEX technology modification, and post-SELEX optimization, the aptamer can be given additional properties, such as the use of chemically produced or modified oligonucleotide libraries to select aptamers with desired features (Green et al., 1995), and the use of chemical modification or base modification can introduce new features into the aptamers (Gold et al., 1995), sometimes aptamer truncations even result in raised affinity (Burke et al., 1996; Ruckman et al., 1998), and fused aptamers could combine different features (Yoshida et al., 2006). But the problem of the requirement for family chemical rapid screening detection remains. For example, family chemical organophosphorus pesticides, which have the toxic effect and chemical structure in common, some of them maybe too low to be detected, but all of them may cause cumulative (additional joint) adverse health effects, so broad-specificity based screening approach with predetermined selectivity is essential to provide an attention for

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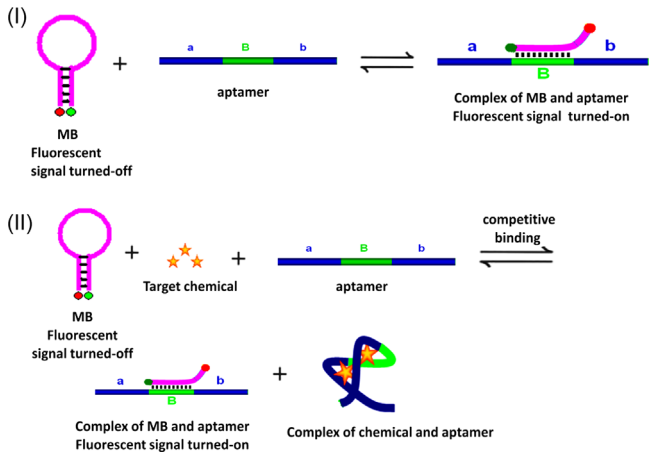
human health benefit. It is possible now to analyze co-existing structures within a population of aptamers with identical sequence (Schroder et al., 1998), but no research has been reported for the simultaneous detection of co-existing chemicals with one broad-specificity aptamer.

Currently, significant interest is focused on development of small molecule aptamers owing to their applications in biosensing, diagnostics, and therapeutics. In spite of the very encouraging promises and great potential for the diverse applications, relatively few aptamers that bind to small molecules have been reported, and methodologies to enhance and broaden their functions by expanding chemical repertories have barely been examined. In a previous study by our group (Wang et al., 2012), aptamers were selected from an immobilized, random single-stranded DNA (ssDNA) library using the SELEX technique, which presented broad specificity for four organophosphorus pesticides recognition. These ssDNAs included primer-binding sequences at both ends and a middle constant sequence, with two random regions of the ssDNA located between the constant sequences; constant middle region of the oligonucleotide library that was used to immobilize the ssDNA library by a partly complementary sequence during the selection of the aptamer (Nutiu and Li, 2005). Herein, in this study, further structural modifications of the aptamers were designed and synthesized with the aim of better characterizing their affinity and specificity for pesticides. Subsequently, a novel competition approach based on a broad-specificity aptamer with increased affinity was explored for simultaneous detection of these substances (Scheme 1), and was successfully exploited using real test sample following sample extraction and cleanup. To our knowledge, this study represents the first report of multi-analysis of organophosphorus pesticides using engineered broad-specificity ssDNA aptamers with a fluorescence signal that is turned on by MB competitive binding, and the developed approach could achieve low-level detection.

2. Experimental

The details of experiments are given in Supplementary material (SI). In brief, aptamer and variants were chemically synthesized; MB 5'-FAM-CTGCACAAGAATCGCTGCAG-DABCYL-3' was used for the competitive binding to aptamers, and the fluorescence intensity changes of MB binding to ssDNA aptamer was calculated as inhibition ratio (IR). Using Visual Molecular Dynamics (VMD) (Hsin et al., 2008; Humphrey et al., 1996) and NAnoscale Molecular Dynamics (NAMD) (Phillips et al., 2005) for aptamer structure modeling and docking, the composited aptamer–ligand structure was energy minimized and equilibrated according to published methods (Bruno et al., 2008), and the Molecular Mechanics – Generalized Born Surface Area (MM – GBSA) method implemented in AmberTool12 was used to calculate the binding energy.



Scheme 1. Strategy for the determination of pesticides: "a" and "b" are the regions of the aptamer derived from the random region of library used during the selection of the aptamer; B present the constant sequences, which can complementary binding to MB. (I) present the control model with chemical absence and (II) present the competition model with chemical presence; compared with the condition (I), the turn-on fluorescent signal in condition (II) will be decreased by the chemical competitively binding to aptamer, and the changes in fluorescence is associated with the changes in the concentration of the chemical.

3. Results and discussion

3.1. Optimization of assay conditions

The details of assay conditions are given in Supplementary material (SI). The strategy for competitive assay development is to incubate the aptamer with the chemical first, and then the MB is added to turn on the signal for detection, and the homogeneous assay is performed at 25 °C for 50 min in aqueous solution of 50 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, and 50 mM Tris/HCl, pH 8.0, with a final acetone content of below 1%.

3.2. Structural modification and characterization

In principle, an aptamer can specifically recognize and bind to a unique ligand, leading to a structural change of an aptamer, which are oligonucleotides (RNA or ssDNA) binding to their target with high selectivity and sensitivity because of their three-dimensional shape, not only by their sequence. DNA aptamers have the capacity to form distinct secondary and tertiary structures that can bind targets with considerable affinity and specificity, structures of aptamer complexes reveal the key molecular interactions conferring specificity to the aptamer–ligand association, including the precise stacking of flat moieties, specific hydrogen bonding, and molecular shape complementarity (Hermann et al., 2000), the stem-loop shows a correlation between sequence structure and function (Nonin-Lecomte et al., 2001). Therefore, certain characteristics of an aptamer can be structurally engineered to create a series of variants or segments, and some may display increased affinity or specificity under multi-characterization, even at the minimum functional molecular size. We have structurally truncated, spliced, and chemically synthesized aptamer variants and segments with the aim of employing a minimum functional structure to examine the correlation between sequence structure and broad-specificity function, and some sequences have been chained together with the intent of extending this broad specificity. The sequences and secondary structures are presented in Supplementary table S2 and Fig. S8. The affinity and specificity of the variants were tested using the targets, which show various dramatically changing affinities and specificities according to the different design strategies employed (Fig. 1).

For the parent aptamer SS2-55, the terminal stem of the stem-loop structure was first shortened by cutting through loop 2–4, with two base pairs of the adjacent loop mutated to induce a destabilized conformation, designated SS2-C-44. This design strategy caused a decrease in the affinity and specificity toward chemicals, particularly the pesticide omethoate, for which only a 5.81% affinity was retained, which implying that the terminal stem and loop 2–4 may play a critical structural role in the broad recognition of chemicals by the aptamer and in specific binding to omethoate, with phorate representing an exception in this case. Next, loop 2–3 was cut, and the remaining SS2-C-29 segment lost almost all affinity and specificity for phorate but retained affinity and specificity for the other chemicals compared with SS2-C-44,

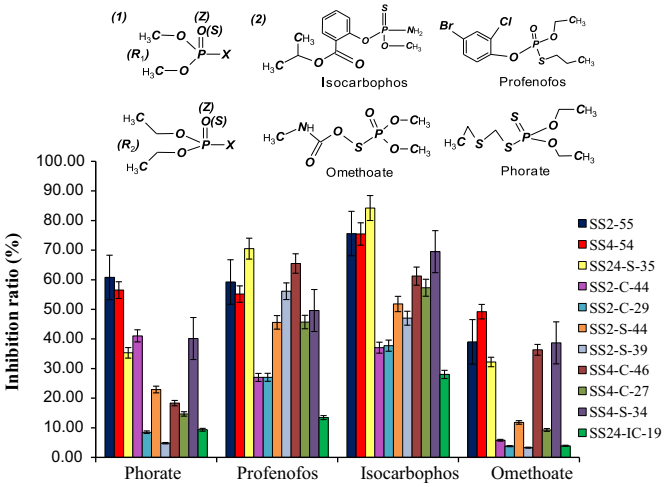


Fig. 1. Affinity and specificity changes of aptamers and recombinant aptamers to the organophosphorus pesticides. (1) General structure of organophosphorus pesticides family. R_1, R_2 present the group of CH_3O or C_2H_5O, Z present oxygen (O) or sulfur (S), X present the Alkoxy group, or Aryloxy or some other groups and (2) structure of DNA binding ligands (chemicals).

which means that loop 2–3 contributes to an important structural function for phorate recognition. Finally, the constant nucleotide sequence SS24-IC-19 was examined, which shows similar characteristics to the SS2-C-29 segment, implying that loop 2(4)-1 may play a key role in the broad recognition of the chemicals phorate, profenofos, and isocarbophos by the aptamer. Our findings indicated that segment SS2-C-29 likely corresponds to the minimum functional size at which broad specificity remains, and the terminal stem and loop 2–4 play a critical structural role in this broad recognition. Thus, the terminal stem and loop 2–4 were spliced to segment SS2-C-29, producing constructs SS2-S-44 and SS2-S-39, which were expected to increase the broad-recognition ability of the segment. The results showed that SS2-S-44 can still carry out the key roles of the aptamer, but SS2-S-39 only increases the affinity and specificity for the chemicals profenofos and isocarbophos. Therefore, the base mutations and base pairs in the adjacent loop need to be examined (Supplementary Fig. S9a).

Different results were found when the selected parent aptamer SS4-54 was subjected to the same modification strategy as the SS2-55 aptamer. When the terminal stem of the stem-loop structure was shortened, the remaining segment, designated SS4-C-46, showed a decreased affinity and specificity for phorate but retained its recognition ability for other chemicals. This result implied that the stem of this aptamer may contribute only to the phorate recognition function, and the other parts of the aptamer may play a critical role in its broad specificity. Next, when loop 4–3 was cut, the resultant segment designated SS4-C-27, showed similar characteristics to segment SS4-C-46, with the exception of a slightly decreased affinity and specificity for omethoate binding. The SS4-C-27 segment therefore appears to represent the minimum functional size of this aptamer. Subsequently, the stem of the parent aptamer and the SS4-C-27 segment were chained together to produce SS4-S-34, which displayed all of the key roles we have described, and the affinity and specificity for all chemicals was increased (Supplementary Fig. S9b).

In summary, for SS2-55, loop 2–4 was found to serve as the shared binding site for four organophosphorus pesticides (particularly for omethoate, which showed stronger binding ability), while loop 2–3 was the site for phorate binding. Loop 2–1 and loop 2–2 represented the shared binding sites for profenofos and isocarbophos. For SS4-54, the nucleotides at the 3' and 5' ends formed a site for phorate and omethoate binding, while loop 4–2 and loop 4–1 were the shared binding sites for profenofos and isocarbophos. Loop 4–3 was a shared binding site for profenofos and omethoate. Some portion of the aptamer might not contribute to chemical recognition but could provide rotational freedom so that the proper position of other functional groups can be retained, such as the constant nucleotide sequences. In addition, differences of a few bases could cause the recognition ability of the aptamer to change dramatically. The various specific contact abilities of the loops could also be affected by the electronic distributions of different chemical groups.

In light of the above findings, based on the critical roles we detected, loop 2–4 was spliced together with a portion of the stem of the SS2-55 parent aptamer (which appears to play an important role in the broad–specificity functions of the SS2-55 parent aptamer) and the SS4-C-27 critical functional segment of the SS4-54 parent aptamer, and the resultant construct was designated SS24-S-35. Surprisingly, this recombinant aptamer showed better affinity for the chemical profenofos, isocarbophos and maintained broad specificity for all chemicals, with only 35 nucleotides remaining, which is much shorter than the parent aptamers (Supplementary Fig. S9c). Further optimization analyses revealed that this recombinant aptamer can function properly under the previously optimized conditions but shows better function under the following conditions: 10 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 50 mM Tris/HCl, pH 8.0. This aptamer was first mixed with chemicals and incubated at 25 °C for 15 min. MB was then added, followed by incubation for an additional 50 min, and fluorescence was recorded thereafter. The conditions for this assay system were as follows: 25 μl aptamer+50 μl chemicals+25 μl MB, and the final concentration of both the aptamer and MB was 100 nM.

3.3. Optimization of aptamer (SS24-S-35)-based competition approach

The details are given in Supplementary material (SI). Based on the assay optimization conducted in Sections 3.1 and 3.2, as a key procedure, the definition of the incubating time of aptamer with chemical was further studied for the quantification. The results showed that the competition involved various processes over time, depending upon the chemicals involved, with the best performance for quantification occurring between 10 and 20 min. At that time, a significant linear relationship was found between the chemical concentration and the changes in fluorescence, and the optimal incubation time was finally determined to be 11 min (Supplementary Fig. S10).

3.4. Real sample detection

The details are given in Supplementary material (SI). In brief, without cleanup, matrix interference greatly affects the fluorescence spectrum of the assay and the MB background fluorescence (Supplementary Fig. S11). After cleanup (Anastassiades et al., 2003), the matrix interference effect was reduced and minimized (Supplementary Fig. S12) with satisfied recoveries (Supplementary Table S3), the limit of quantification (LOQ) for phorate, profenofos, isocarbophos, and omethoate could achieve 19.2, 13.4, 17.2, and 23.4 nM (0.005 mg L^{-1}) in the range of 0.01–10.0 mg kg^{-1}, correspondingly (Supplementary Fig. S13 and Table S4).

3.5. Modeling and molecular docking (SS24-S-35 aptamer)

The details of modeling and molecular docking are given in Supplementary material (SI). The three-dimensional (3-D) model shown in Fig. 2 revealed a two-and-a-half helix structure with three potential binding sites for ligands, which located in the minimum

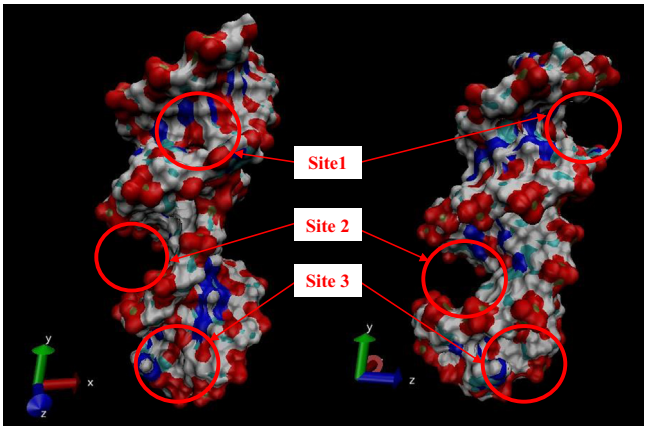


Fig. 2. The 3-D structure modeling of aptamer SS24-S-35 and it is possible binding sites.

functional structures of parent aptamers. It was found that several types of forces, including hydrogen bonds and van der Waals interactions appear to play a significant role in enabling and stabilizing the binding of chemicals to the aptamer (Supplementary Table S5).

4. Conclusion

An engineered broad-specificity aptamer based competitive approach for the detection of organophosphorus pesticides was developed in this study, which could simultaneously detect the co-existing chemicals. Aptamer modifications indicate that the locations of functional groups (loops) are associated with various specific contacts with different target chemicals that could vary the affinity of the binding of the aptamer to chemicals. Concerning the aptamer's broad recognition ability, the overall three-dimensional pattern of the structure and loops could participate in architectural recognition, and further modifications of DNA base, sugar, phosphate and the base pairs present in the adjacent loop (Pinheiro et al., 2012; Lato et al., 2002), which is an essential nucleation point for functional participation in the interaction, should be further performed.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.12.020.

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