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Multiple GO-SELEX for efficient screening of flexible aptamers†

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We describe a simple, high-speed, high-throughput aptamer screening for a group of small molecules using graphene oxide (simple Multi-GO-SELEX) without immobilizing targets. The affinities of ten different ssDNA aptamers successfully obtained for three pesticides were in the range of 10–100 nM. Besides a specific aptamer for each target, we found a couple of flexible multi-target aptamers, which can bind with 2 or 3 different molecules. These flexible aptamers developed for binding with a mixture of targets are not only significant for the rapid screening of a group of small molecules but also offer great promise for aptamer-based biosensor applications.

Pesticides are widely used in agriculture to control insects, microorganisms, fungi, weeds, and other pests in order to preserve the crops.[1] However, pesticides can have deleterious effects and are toxic to humans and animals, because they have been known to be carcinogenic, mutagenic, or hormone mimickers.[2] Some compounds have the ability to persist for a long time in the environment, including, being in soil, water, and air, even though they reside inside the seeds. Recently, a few studies have suggested that pesticide exposure causes Parkinson's disease and neurotoxicity.[3–5] Thus, the evaluation and estimation of the quantity of pesticide compounds in the environment are very important.[6] There is a limited number of research done in the field of sensors available based on the DNA aptamer for pesticides.[7] Systematic evolution of ligands by exponential enrichment (SELEX)[8] is a well-known process to isolate these nucleic acid sequences, which functionally bind and discriminate their target in a complex heterogeneous environment. Aptamers are not only inexpensive and chemically synthesized but are also thermostable, with high affinity and specificity.[9–13] Due to inefficiencies and low quality of aptamers, the number of publications related to the generation of aptamers is limited.[14,15] Recently, we have developed a GO-SELEX method for both protein and virus particles,[16,17] which is simple to use, cost effective and an immobilization-free platform for screening of aptamers that bind to their target with high affinity and specificity. Herein, we report a simple, high-speed, high-throughput aptamer screening method for a group of small molecules using graphene oxide (simple Multi-GO-SELEX) without immobilizing targets. In this simple Multi-GO-SELEX, we chose 3 difference types of pesticides: tebuconazole as a plant growth regulator class, inabenfide as plant pathogenic fungi, mefenacet as a herbicide class, and the aptamers for these pesticides were successfully obtained. Their specificities were estimated based on both the gold nanoparticle colorimetry and ITC assays.[18] More interestingly, besides a specific aptamer for each target, we found 2 flexible multi-target aptamers, which can bind to 2 or 3 targets flexibly, such as T4 and T2 aptamers for tebuconazole, inabenfide and mefenacet. Unlike the previous study, in which a set of specific aptamers was used to increase the binding efficiency and the sensitivity with a target,[19] a flexible multi-target aptamer could bind to several main targets in this study. Therefore, these flexible multi-target aptamers are more simple, fast, and convenient for detecting and screening a group of small molecules.

In this simple Multi-GO-SELEX illustrated in Scheme 1, the counter targets were mixed with the ssDNA random library to eliminate false positive binding of aptamers to the counter targets as the 1st step. In this GO counter-SELEX step, six different counter targets were chosen and the counter-SELEX step was employed to remove the non-specific aptamers or just ssDNAs which may be present in the DNA pool and bind to the related structures similar to main targets. Thereafter, 100 μl of 5 mg ml^{-1} GO solution was added into the mixture for 1 hour incubation and then ssDNA-bound counter targets and the remaining counter targets were discarded by centrifugation. The ssDNA bound GO was incubated with the mixture of tebuconazole, inabenfide and mefenacet for de-adsorption of ssDNAs from GO to take ssDNAs bound to the targets back to solution. The concentration of each main target in the mixture was optimized to 200 pmole for maximum affinity binding between ssDNAs and the target. When the targets come close to the surface of GO where the

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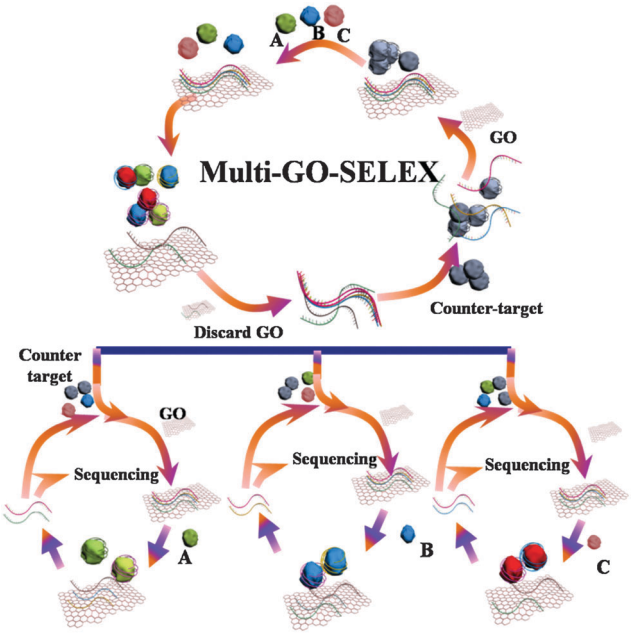

Scheme 1 Schematic illustration of multi-GO-SELEX. In multi-GO-SELEX, the 3 main targets are added into ssDNA-bound GO; the ssDNA are desorbed by main targets and they are recovered, amplified and purified for next rounds.

ssDNA aptamer candidates are bound, the structure of these ssDNAs with affinity to the targets are reformed and so the π–π stacking interactions with the GO surface are broken to refold their conformation, and result in the release from GO. After incubation for desorption of aptamers, other steps such as PCR and PAGE are performed to produce refined ssDNAaptamer candidates for the next round or sequencing.

The percent recovery of ssDNAs bound to the mixture of main targets from the pool of the ssDNA random library dramatically increased from the 1st round (4.3%) to the 3rd round (63%) (Fig. 1). The 4th and 5th GO-SELEX rounds were carried out individually for each different target. It is not surprising that the percent recovery in the 4th round dropped down to 29%, equivalent to 1/3rd of the resulting quantity in the 3rd round, due to the DNA sequence population in the

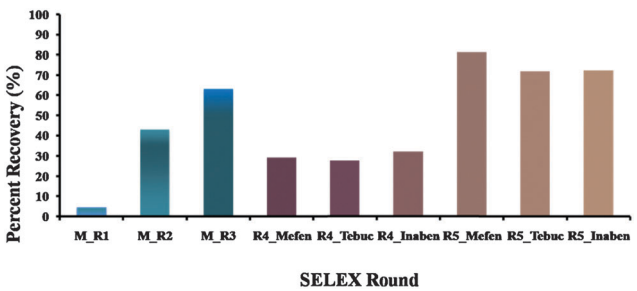

Fig. 1 Percent recovery of the target-bound ssDNA library from the pool of the ssDNA random library. After the 3rd round of the Multi-GO-SELEX process, the 3 main targets are separated during SELEX in further rounds and results have shown that the percent recovery of target-bound ssDNA is saturated after the 5th round of SELEX, respectively.

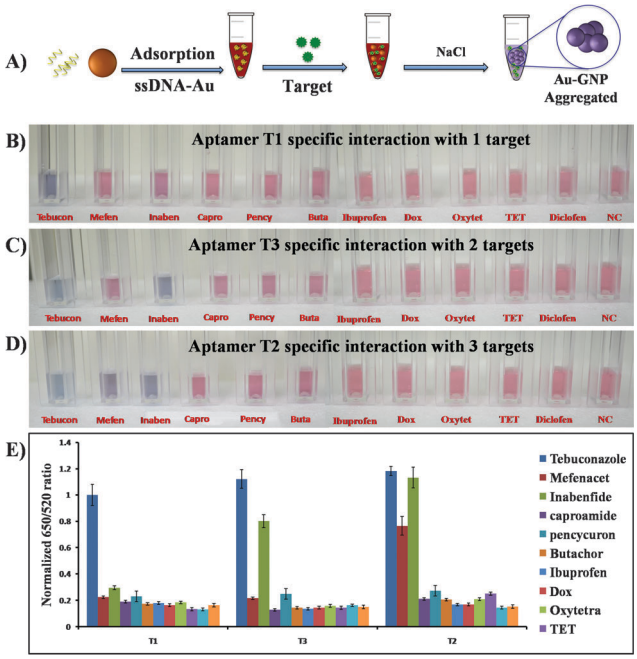

Fig. 2 The specificity test by colorimetric assay. (A) The images of specific interaction of various pesticides and small molecules with (B) aptamer T1, (C) aptamer T3, and (D) T2, respectively, (E) the normalized 650/520 ratio of AuNP in colorimetric assay with tebuconazole, mefenacet, inabenfide, caproamide, pencycuron, butachor, ibuprofen, dox, oxytetracycline, tetracycline and diclofenac.

random library which was enriched with a sequence specific to each target in the mixture. After the 5th round of GO-SELEX, the enriched ssDNAs were cloned and sequenced, and 10 sequences were collected and their Gibbs free energy was estimated (see Table S1 in ESI†).

After Multi-GO-SELEX screening, the affinity of aptamers obtained for three main targets, respectively, was characterized. The specificity of the aptamer candidates was initially determined by Au-NP colorimetric assay[20,21] and three aptamer sequences showed their specificity to each target (Fig. 2A and B and see ESI,† Fig. S5–S10). The binding strength between the candidate aptamer and the target was evaluated by ultrasensitive Isothermal Titration Calorimetric (ITC) assay. To analyze Kd, the binding curve obtained from the ITC assay was fitted by the single site binding model to determine the association constant. The high affinity binding between aptamer T1 and tebuconazole exhibited a Kd value of 1 nM (Fig. 4A and B) and binding strengths of all aptamers with targets were in the range of 1–100 nM (see ESI,† Fig. S18). The results showed that the binding strength of aptamers with targets was as low as the 10 nM range, and this is another confirmation of the affinity binding between the aptamer and the target, in addition to the colorimetric assay method.

In the dose dependent experiments, the limit of detection for a specific pesticide with its corresponding aptamer was determined after mixing with various concentrations of the target by allowing the complex of an aptamer and Au-NP in the colorimetric assay (Fig. 3A). In most cases (Fig. 3B–D and see ESI,† Fig. S11–S17) the detection limit of the tebuconazole, mefenacet and inabenfide, respectively, were in the range of 100 to 400 nM. As shown in

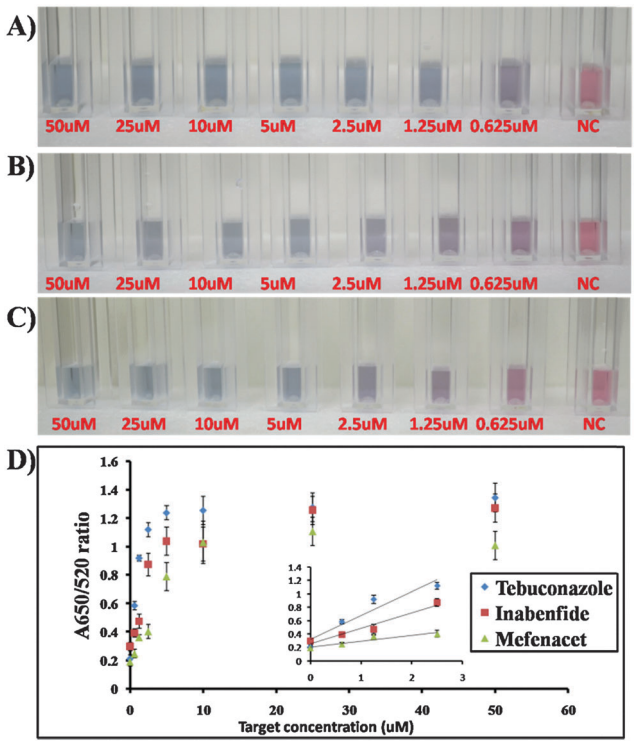

Fig. 3 The sensitivity and dose dependent assay. The sensitivity result of multi-target aptamer (T2) with (A) tebuconazole, (B) inabenfide and (C) mefenacet, (D) the detection limit of the T1 aptamer bound with tebuconazole, inabenfide and mefenacet, respectively, by colorimetric assay (inset: enlarged spectrum analysis graph with a target concentration from 0 to 2.5 μM).

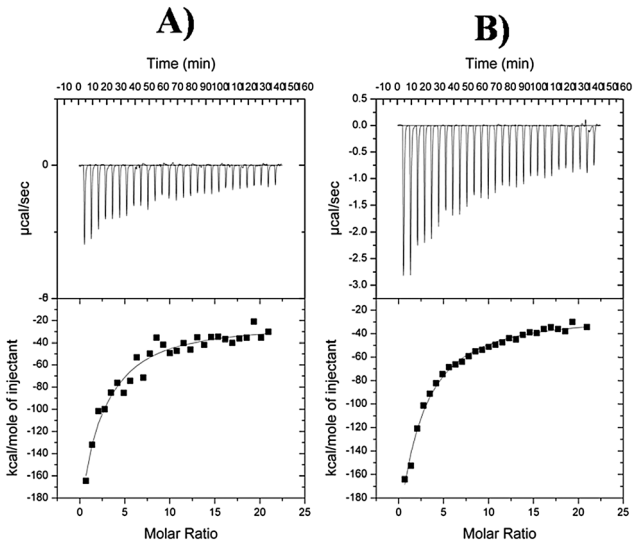

Fig. 4 (A) Molecular interactions of the T2 aptamer and tebuconazole measured by ITC analysis with 50 mM Tebuconazole titrated into 0.025 mM T2 aptamer. (B) Molecular interactions of the T2 aptamer and Inabenfide measured by ITC analysis with 50 mM Inabenfide titrated into 0.025 mM T2 aptamer.

Fig. 3D, the absorbance ratios of 650 mn/520 mm were plotted against tebuconazole, mefenacet and inabenfide concentrations. It is clear from these results that the pesticide compounds can be detected up to as low as 128 nM of tebuconazole, 276 nM of mefenacet and 191 nM of inabenfide.

Notably, we found the aptamers which can bind to 2 or 3 different targets, respectively (Fig. 2C and D). It has been considered initially that this phenomenon was a result of a non-specific binding of target and aptamer candidates. However, when we carried out the same experiment with other counter targets, they showed specific binding only with their own targets (Fig. 2E). In addition, the colorimetric assay for a scrambled sequence of aptamer T2, denoted T2 scrambled No. 30 (T2S-No. 30), was studied under the same conditions. In the test, the scrambled T2S-No. 30 did not show any color changes upon the addition of not only three main different targets but also other counter targets, meaning that the aptamers selected in this study are specific to only main targets (Fig. S21 in ESI†).

Therefore, the mechanism of this Multi-SELEX method can be understood as follows. The pool of the random library probably has some ssDNAs which have a flexible conformation. By mixing the targets in the same pool of ssDNAs, a chance for these ssDNAs, flexible aptamers, binding with multi-targets was increased and screened in the process of Multi-SELEX. In contrast to the uncontrollable non-specific binding aptamers which bound with their counter targets, in addition to the main target, we can control and obtain these flexible aptamers for a group of small molecules by mixing in the Multi-GO-SELEX process. These flexible aptamers were shown to be specific only with the main targets. In this study, we used 56 mer of random library ssDNA for the Multi-SELEX pool because of the size of target molecules. Besides the 56 mer candidate aptamers obtained from Multi-GO-SELEX, we also got the 76 mer aptamer candidates which showed the affinity binding with our target because they are selected during the SELEX process.

To study the kinetic affinity binding of these flexible multi-target aptamers with a mixture of targets, the colorimetric assay for the mixture of targets was carried out (Fig. 5A).

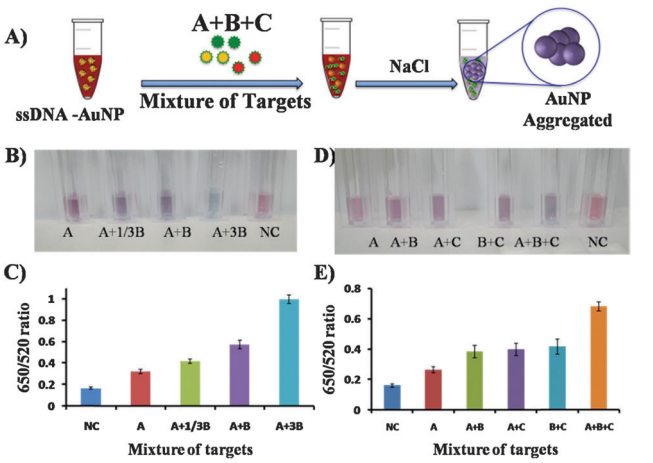

Fig. 5 The binding affinity of flexible multi-target aptamers with a mixture of targets. (A) The scheme of AuNP colorimetric assay. (B and C) The image and graph of the binding affinity of flexible aptamer T3 with a mixture of Tebuconazole and Inabenfide with ratios 1 : 0; 1 : 1/3; 1 : 1 and 1 : 3, respectively. (D and E) The image and graph of the binding affinity of flexible aptamer T2 with a mixture of tebuconazole, mefenacet and inabenfide with ratios 1 : 0 : 0; 1 : 1 : 0; 1 : 0 : 1; 0 : 1 : 1 and 1 : 1 : 1. The concentration of tebuconazole is fixed at 500 nM. A-tebuconazole, B-inabenfide, C-mefenacet.

Prior to the mechanism of flexible aptamer binding, we used the concentration of tebuconazole of 500 nM for all experiments and changed the concentration of other targets. For example, in the case of aptamer T3, which can bind with two targets, the mixture of tebuconazole and inabenfide at various ratios 1 : 0; 1 : 1/3; 1 : 1; 1 : 3 was added in the solution of AuNP–flexible T3 (Fig. 5B and C). The results showed that the absorbance ratios at 650–520 nm increased from 2.8 to 1.1 when the ratio of tebuconazole and inabenfide was changed from 1 : 0 to 1 : 3 by the addition of inabenfide. The same pattern was obtained in case of flexible aptamer T2, which can bind to three targets, tebuconazole, inabenfide, and mefenacet, the absorbance ratios were greatly changed from 2.9 for a single target, 4.2 for the mixture of 2 targets, and 6.8 for the mixture of 3 targets, respectively, at the ratios of 1 : 0 : 0, 1 : 1 : 0, 1 : 0 : 1, 0 : 1 : 1 and 1 : 1 : 1 (Fig. 5D and E). In both cases of aptamer T3 and T2, there is a significant increase in the absorbance ratio (A650/A520) when the ratio of tebuconazole with inabenfide or mefenacet is changed from 1 : 0 to 1 : 3 by adding inabenfide and/or mefenacet. The results of kinetic affinity binding of flexible multi-target aptamers indicate that the color change of AuNPs is enhanced when more amount of target was added in the AuNP–flexible aptamer mixture. In other words, in the presence of target-induced conformation, the flexible aptamers can refold their structures to bind to each of the different targets, respectively. This explanation was confirmed again by measuring Circular Dichroism (CD) changes. Upon the addition of each different main target in the solution containing aptamer T2, similar CD patterns were obtained along with similar peak shifting with increased ellipticity (see ESI,† Fig. S22).

In summary, to the best of our knowledge, we report, for the first time, a simple, cost-effective and rapid Multi-SELEX method for a small molecule target. We found a simple way to obtain aptamers, not only for a single target but also for a group of targets. Therefore, it may reduce the cost effectively and shortens SELEX time. Remarkably, the flexible aptamers which were obtained together with a single target aptamer simultaneously could be useful in the strategy for screening aptamers for multi-targets, especially, toxic molecules.

Our technology suggests that the simultaneous screening of multiple target aptamers will provide opportunities for aptamer discovery, in molecular diagnostics and biotechnology.

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