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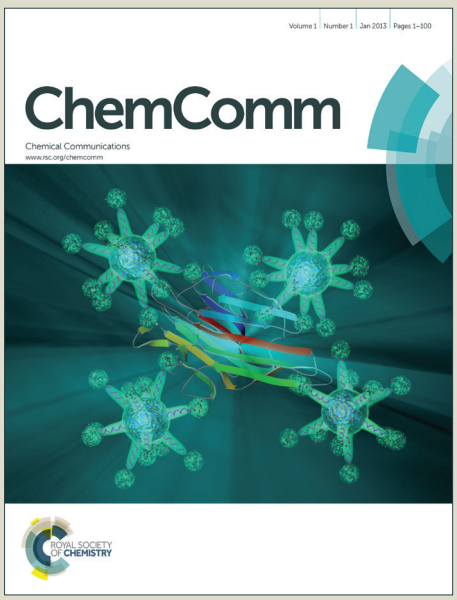

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Multiple GO-SELEX for Efficient Screeningof Flexible Aptamers

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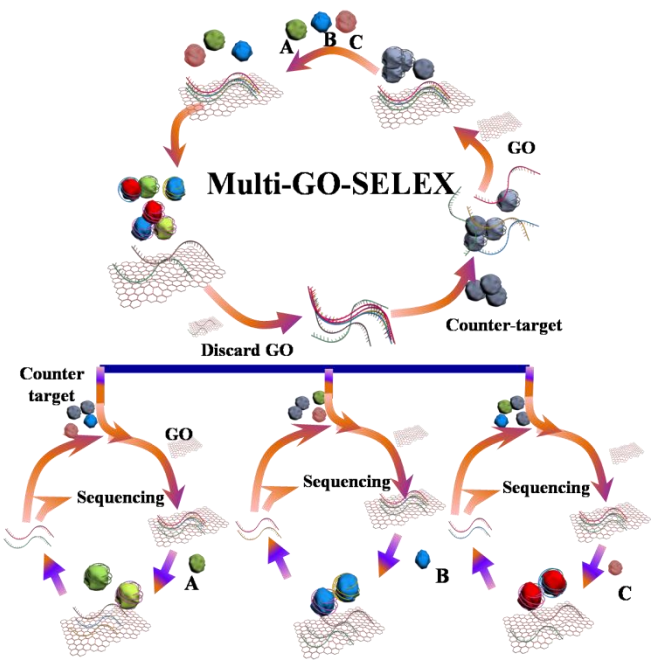
We describe a simple, high-speed, high-throughput aptamer screening for a group of small molecules using Graphene Oxide (simple Multi-GO-SELEX) without immobilizing targets. The affinities of ten different ssDNAaptamers successfully obtained for three pesticides were in a range of 10~100 nM. Beside a 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 not only are 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 is related to cause Parkinson's disease and neurotoxicity.[3- 5] Thus, the evaluation and estimation of the quantity of pesticide compounds in environment are very important.[6] There is a limited number of research done on the field of sensor available based on DNA aptamer for pesticide.[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. Aptamer are not only inexpensive and chemically synthesized but also are thermostable, with high affinity and specificity.[9- 13] Due to its inefficiencies and low quality of aptamers, the number of publications related to the generation of aptamers are limited.15, 16 Recently, we have developed a GO-SELEX method for both protein and virus particles,[16, 17] which is simple to use, cost effective, 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 Plant Growth Regulator class, Inabenfide as plant pathogenic fungi, Mefenacet, as herbicide class, and the aptamers for



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

these pesticides were successfully obtained. Their specificities were estimated based on the both gold nanoparticle colorimetry and ITC assays.18 More interestingly, beside 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 aptamer 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 for 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, the 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 DNA pool and bind to the related structures similar to main targets.Thereafter, 100ul of 5mg/ml GO solution was added into the mixture for 1 hour incubation and then ssDNA-bound counter targets and the remained counter targets were discarded by centrifugation. The ssDNA bounded GO was incubated with the mixture of tebuconazloe, 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 mixture was optimized to 200 pmole for maximum affinity binding between ssDNAs and the target. When the targets come close to surface of GO where the ssDNAaptamer candidates are bounded on it, 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, PAGE are performed to produce a refined ssDNAaptamer candidates for next round or sequencing.

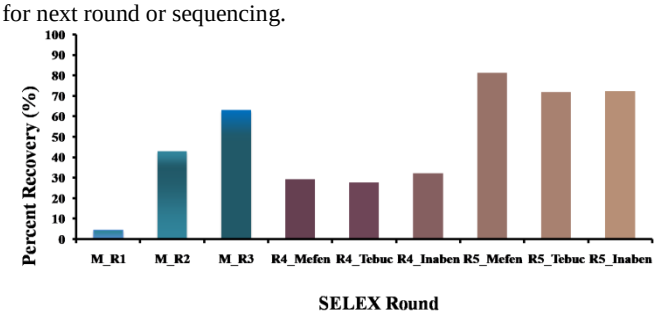


Figure 1. Percent recovery of target-bounded ssDNA library from pool random library ssDNA. After 3rd round of Multi-GO-SELEX process, the 3 main targets are separated during SELEX in further rounds and results have shown that the percent recovery of target-bounded ssDNA is saturated after 5th round of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

The percent recovery of ssDNAs bound to the mixture of main targets from the pool of ssDNA random library is dramatically increased from 1st round (4.3%) to 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 of 4th round is dropped down to 29% recovery, equivalent of 1/3 of the resulting quantity of the 3rd round, due to the DNA sequence population in the random library which was enriched with sequence specific to each target in the mixture. After 5th round of GO-SELEX, the enriched ssDNAs were cloned and sequenced, and 10 sequences were collected and their Gibb's free energy were estimated (**see Table S1 in ESI†**).

After multi-GO-SELEX screening, the affinity of aptamers obtained for three main targets, respectively, were characterized. The specificity of the aptamer candidates was initially determined by Au-NP colorimetric assay[20, 21] and three aptamer sequences showed its specificity to each target (**Fig. 2A&B and see ESI† Figures S5-S10**). The binding strength between the candidate aptamer and the target was evaluated by ultrasensitive Isothermal Titration Calorimetric (ITC) assay.[22]ToanalyzeKd, the binding curve obtained from the ITC 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, 4B**) and all binding strength of aptamers and targets were in a range of 1~100 nM (**see ESI† Figures S18**). The results showed that the binding strength of aptamers with targets was as low as 10 nM range, and this is an another confirmation of the affinity binding between the aptamer and the target, in addition to the colorimetric assay method.

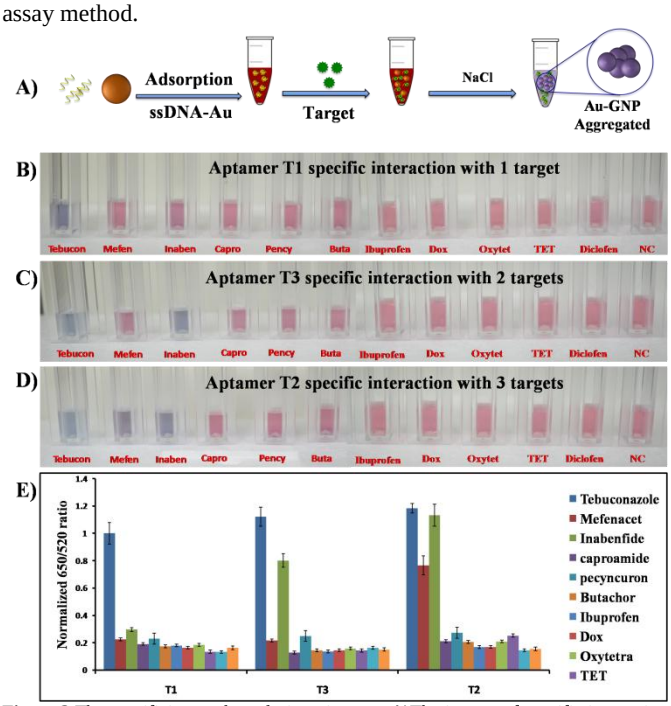


Figure 2. The specificity test by colorimetric assay.A)The images of specific interaction of various pesticides and small molecules with B) Aptamer T1, C) aptamer T3, and D) T2 respectively, E) The normalized 650/520 ratio of AuNP in colorimetric assay with tebuconazole, mefenacet, inabenfide , caproamide,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

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 aptamer and Au-NP in the colorimetric assay (**Figure 3A**). In most cases (**Figure 3B-3D and see ESI† Figures S11-S17**) the detection limit of the tebuconazole, mefenacet and inabenfide, respectively, were in a range of 100 to 400nM. As shown in Fig. 3D, the absorbance ratios of 650mn/520mm of 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&2D**). It has been considered initially that this phenomenon was a result from a non-specific binding of target and aptamers candidate. However, when we carried out the same experiment with other counter targets, they showed specific binding only with its own targets (**Fig2E**).In addition, the colorimetric assay for a scrambled sequence of aptamerT2, named T2 scrambled No. 30 (T2S-No30), was studied in the same condition. In thetest,the scrambled T2S-No30 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 followings. In the pool of the random library, it

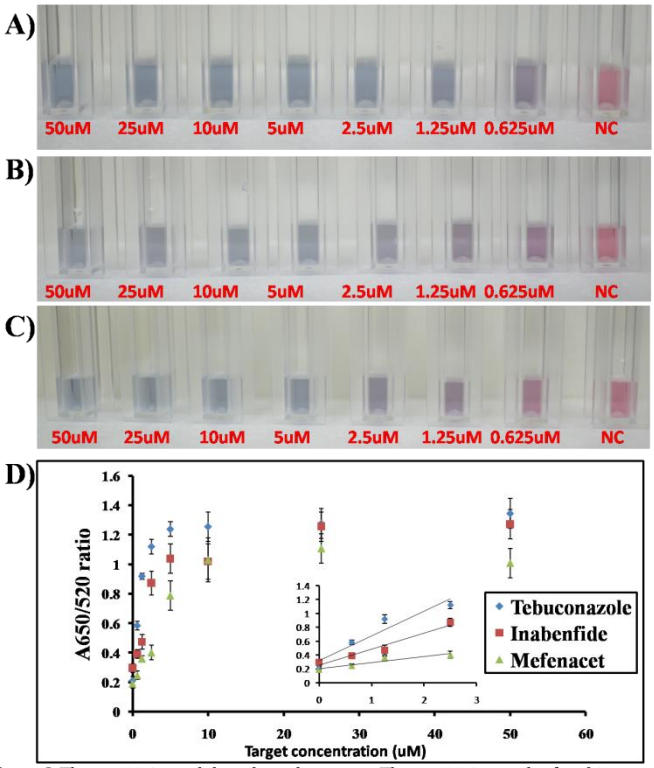


Figure 3. The sensitivity and dose dependent assay. The sensitivity result of multi-target aptamer (T2) with A) Tebuconazole, B) Inabenfide and C) mefenacet, D) The detection limit of T1 aptamer bind with Tebuconazole, Inabenfide and mefenacet respectively by colorimetric assay (Inset: enlarged spectrum analysis graph with target concentration from 0 to 2.5uM).

probably has some ssDNAs which have flexible conformation. By mixing the targets in the same pool of ssDNAs, a chance for these ssDNAs, fexibleaptamers, binding with multi-targets were increased and screened in the process of Multi-SELEX. In contrast to the

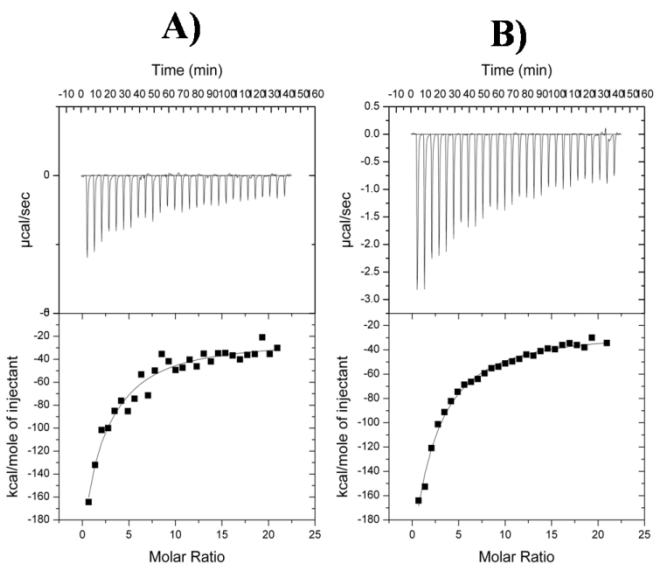


Figure 4. A) Molecular interactions of the T2 aptamer and Tebuconazole measured ITC analysis with 50mM Tebuconazole titrated into 0.025mM T2 aptamer. B). Molecular interactions of the T2 aptamer and Inabenfide measured ITC analysis with 50mM Inabenfide titrated into 0.025mM T2 aptamer

uncontrollable non-specific binding aptamers which bound with its 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 multi-GO-SELEX process. These flexible aptamers were shown to be specific only with the main targets.In this study, we used 56mer of random library ssDNA for Multip-SELEX pool because of the size of targets molecules. Beside the 56mer candidate aptamers are obtained from Multi-GO-SELEX, we also got the 76mer aptamer candidates which are showed the affinity binding with our target due to they are selected during SELEX processing.

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 (**Figure 5A**).

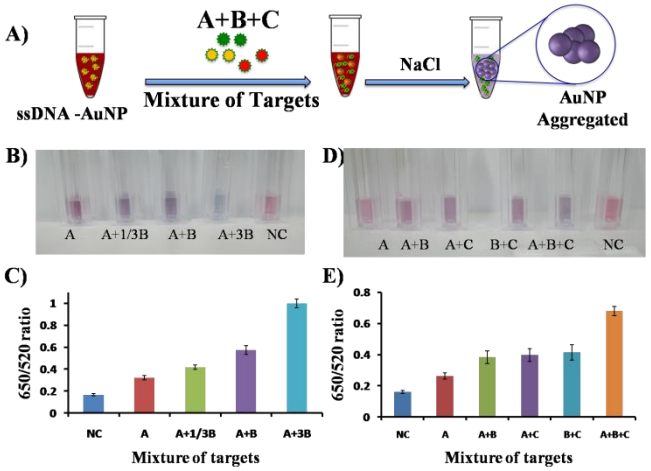


Figure 5. The binding affinity of flexible multi-target aptamers with a mixture of target. A) The scheme of AuNP colorimetric assay. B& C) The image and graph of the binding affinity of Flexible aptamers T3 with mixture of Tebuconazole and Inabenfide with ratio 1:0; 1:1/3; 1:1; 1:3, respectively. D&E) The image and graph of the binding affinity of Flexible aptamers T2 with mixture of Tebuconazole, mefenacetand Inabenfide with ratio 1:0:0; 1:1:0; 1:0:1; 0:1:1; 1:1:1. The concentration of Tebuconazole is fixed at 500nM. A-Tebuconazole, B-Inabenfide, C-Mefenacet.

Prior to the mechanism of flexible aptamer binding, we used the concentration of Tebuconazole at 500nM for all experiments and changed at the difference concentration of other targets. For example, in case of aptamer T3, which can bind with the two targets, the mixture of Tebuconazole and Inabenfide at various ratio 1:0; 1:1/3; 1:1; 1:3 was added in the solution of AuNP-flexible T3 (**Figure 5B&C**).The results showed that the absorbance ratios at 650–520 nm were increased from 2.8 to 1.1 when the ratio of Tebuconazole and Inabenfide were changed from 1:0 to 1:3 by the addition of amount 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(**Figure 5D&E**). In both cases of aptamer T3 and T2, there are significant increasing in the absorbance ratio (A650/A520) when the ratio of tebuconazole with inabenfide or mefenacet are 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 are enhanced when more amount of target were added in AuNP/Flexible aptamer mixture. In other words, in the present of targets-induced conformation, the flexible aptamers can refold their structures to bind each of different targets, respectively. This

explanation was confirmed again by measuringCircular Dichroisms (CD) changes. Upon the addition of each different main target in the solution containingaptamer T2, similar CD patterns were obtained along with similar peak shifting with increased ellipicity(**see ESI† Figures 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 small molecule target. We found a simple way to obtain aptamers, not only for a single target but also for group of targets. Therefore, it may reduce the cost effectively and shortens SELEX time. Remarkably, the flexible aptamers which were obtained together with single target aptamer simultaneously could be useful strategy for screening aptamers for multi-targets, especially, toxic molecules.

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

Notes and references

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