



Aptamer-based microcantilever-array biosensor for profenofos detection

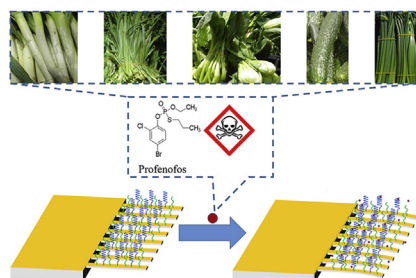
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HIGHLIGHTS

- An aptamer-based microcantilever array sensor in stress mode was developed to detect profenofos.
- Aptamers are ideal recognition molecules for microcantilever platform.
- The detection limit is low to 1.3 ng mL^{-1} , which outperforms other aptamer-based detection method.
- The direction of microcantilever deflections accord with theoretical analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Profenofos, a highly poisonous organophosphorus pesticide, has been widely used in agricultural production. These pesticide residues have seriously influenced food security and threatened human health, and new methods with high sensitivity are greatly needed to detect profenofos. Here, we developed an aptamer-based microcantilever-array sensor operated in stress mode to detect profenofos, with advantages of being a label-free, highly sensitive, one-step immobilization method capable of quantitative and real-time detection. The microcantilevers were functionalized with a profenofos-specific aptamer (SS2-55), and then the specific binding of profenofos to aptamer induced a deflection of the microcantilever, which was monitored using an optical method in a real-time manner. The microcantilever deflection showed a positive relationship with profenofos concentration, and the detection limit was low to 1.3 ng mL^{-1} (3.5 nM) for profenofos, which was much lower than other aptamer-based detection methods. The selectivity of the sensor was verified with another organophosphorus pesticide. Additionally, we successfully detected profenofos dissolved in vegetable-soak solution. Our results showed that this aptamer-based microcantilever-array sensor is a convenient and label-free method for detecting profenofos in small amounts and has great potential for food-security applications.

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1. Introduction

Organophosphorus pesticides are highly effective pesticides and herbicides widely used in agricultural settings for crop-pest control and prevention [1,2]. Because of their wide use and high toxicity, the threat of organophosphorus pesticide residues, and particularly those of highly poisonous organophosphorus pesticides, such as

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phorate, profenofos, isocarbophos, and omethoate, to the environment and human health has increased in recent years [3,4]. Small amounts of these pesticide residues can significantly damage human health [5]. The Chinese national standard (GB 2763-2016) stipulates that the maximum residue limit for profenofos in cereal is 0.02 mg kg^{-1} , and that the maximum residue limit for phorate in vegetables and fruits is 0.01 mg kg^{-1} .

Current analytical methods for detection of these organophosphorus pesticides include gas chromatography (GC), high-performance liquid chromatography (HPLC), enzymatic inhibition methods (EIMs), and enzyme-linked immunosorbent assay (ELISA) [6]. GC [7] and HPLC [8] are sensitive and can achieve quantitative detection; however, these two techniques normally require expensive equipment, extensive sample pretreatment, and complex procedures. Additionally, samples need to be transformed into a gas for GC, which will cause partial sample decomposition and decrease sensitivity. EIMs [9,10] utilize the inhibitory effect of organophosphorus pesticides to the enzyme, making it a rapid and simple method; however, it lacks specificity and sensitivity. ELISA [11,12] is highly sensitive and has good specificity, but requires labeling of the molecules, which is difficult for small molecules and might affect detection sensitivity. Generally, organophosphorus pesticides are small molecules, with molecular weights on the order of hundreds of Daltons. Moreover, organophosphorus pesticides might not have the ability to independently stimulate humans or animals to produce antibodies, requiring conjugation with carrier proteins for this to occur [13]. Therefore, antibody preparation for small molecules is complex and difficult, making the development of sensitive and convenient techniques to detect these pesticide residues highly important.

In recent years, research into the development of microcantilever-biosensor techniques has increased due to their high sensitivity, rapid responses, label-free requirements, and allowance for real-time detection [14,15]. Recognition molecules are first immobilized on one side of the microcantilever, and for microcantilevers operated in stress mode, the reaction between the recognition and target molecules causes a change in surface stress that induces microcantilever deflection [16]. This technique has been widely used for the detection of heavy metal ions [17,18], drugs [19–21], antibiotics [22], explosive vapors [23], and mycotoxins [24], and it was also applied to detect pesticide residues using antibodies as recognition molecules [16,25]. For microcantilevers operated in dynamic mode, the binding of target molecules to recognition molecules adds an additional mass to the microcantilever, resulting in a decreased resonance frequency. Generally, the dynamic mode is superior for detecting macromolecules [26,27].

Aptamers are single-stranded oligonucleotide sequences that can be selected from the systematic evolution of ligands by exponential enrichment (SELEX) process [28]. Aptamers can conjugate with various kinds of target molecules with high affinity and specificity, similar to antigen–antibody interactions. In comparison to antibodies, aptamers own numerous advantages. It is low-cost and convenient for aptamers to synthesis, and various chemical tags can be easily attached to aptamers without influencing their target affinity, which is a desirable property for recognition molecule. Moreover, aptamers exhibit superior stability, high specificity, and sensitivity [29]. With these advantages, aptamers constitute an ideal probe for detecting small molecules [30]. As recognition molecules, aptamers have successfully been applied for the detection of heavy metal ions [31,32], microorganisms [33,34], cells [35] and antibiotics [36,37]. A screening process for aptamers capable of binding to organophosphorus pesticides was previously reported [38]. Pesticide-specific aptamers have been used for the detection of corresponding organophosphorus pesticides using fluorescence

methods [39–41]. The fluorescence method has been widely applied with high sensitivity, but fluorescent labels or molecular beacons are required.

Microcantilever-array sensors can overcome the disadvantages associated with these methods; however, there remains no report of an aptamer-based detection method for organophosphorus pesticides using a microcantilever platform. For microcantilever sensors, the functionalization process affects detection stability and sensitivity, and for the aptamers, thiolation can be accomplished during synthesis. Therefore, the functionalization of a microcantilever with an aptamer is very convenient. Moreover, aptamers are more chemically stable than antibodies, which is important to detection sensitivity. Therefore, an aptamer-based microcantilever sensor might be ideal for rapid and sensitive detection of organophosphorus pesticides.

In this article, we developed an aptamer-based microcantilever-array sensor to detect profenofos. The microcantilever array was modified with a thiolated profenofos-specific aptamer, followed by its detection of different concentrations of profenofos in stress mode. To investigate its specificity, we detected ethyl parathion using microcantilever array functionalized with profenofos-specific aptamer, and a microcantilever array lacking an aptamer was used to detect high concentrations of profenofos. These experiments confirmed the specificity of the method. Additional results showed that the microcantilever array successfully detected profenofos dissolved in vegetable-soak solution.

2. Materials and methods

2.1. Reagents and solutions

Profenofos-specific aptamer [SS2-55; 5'-HS-(CH₂)₆-AAG CTT GCT TTA TAG CCT GCA GCG ATT CTT GAT CGG AAA AGG CTG AGA GCT ACG C-3'] was synthesized by Sangon (Shanghai, China). Profenofos, ethyl parathion, and 6-mercapto-1-hexanol (MCH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris-HCl, KCl, and MgCl₂·6H₂O were supplied by Sangon, and all other chemicals were of analytical reagent grade and obtained from Beijing Chemical Reagents Company (Beijing, China).

The aptamer was dissolved in solution containing 300 mM NaCl, 50 mM KCl, 10 mM MgCl₂, and 50 mM Tris-HCl (pH 8.3) prior to use. The buffer used in the experiments was Tris-buffer containing 50 mM NaCl, 10 mM KCl, 10 mM MgCl₂, and 50 mM Tris-HCl (pH 8.0) [41].

2.2. Apparatus

The 96-well polystyrene microplates were purchased from Costar (Corning, NY, USA). Microcantilever arrays were purchased from Micromotive GmbH (Mainz, Germany). Every microcantilever array contained eight silicon microcantilevers, with one side of the microcantilever having a thin film of titanium (2 nm) covered with a 20-nm layer of gold. The dimensions of the microcantilever were $500 \mu\text{m} \times 90 \mu\text{m} \times 1 \mu\text{m}$.

We used a self-developed microcantilever-array instrument based on optical fiber for microcantilever-array sensor detection (Fig. 1) [42]. The array was placed into a fluid cell with a temperature-control device. Eight optical fibers were coupled to lasers and emitted eight parallel laser beams that sequentially illuminated microcantilevers in an array, and laser beam was reflected from the tip of the microcantilever. The position of the laser beam was monitored with a position sensitive detector (PSD). An injection pump was used to control the flow of liquid.

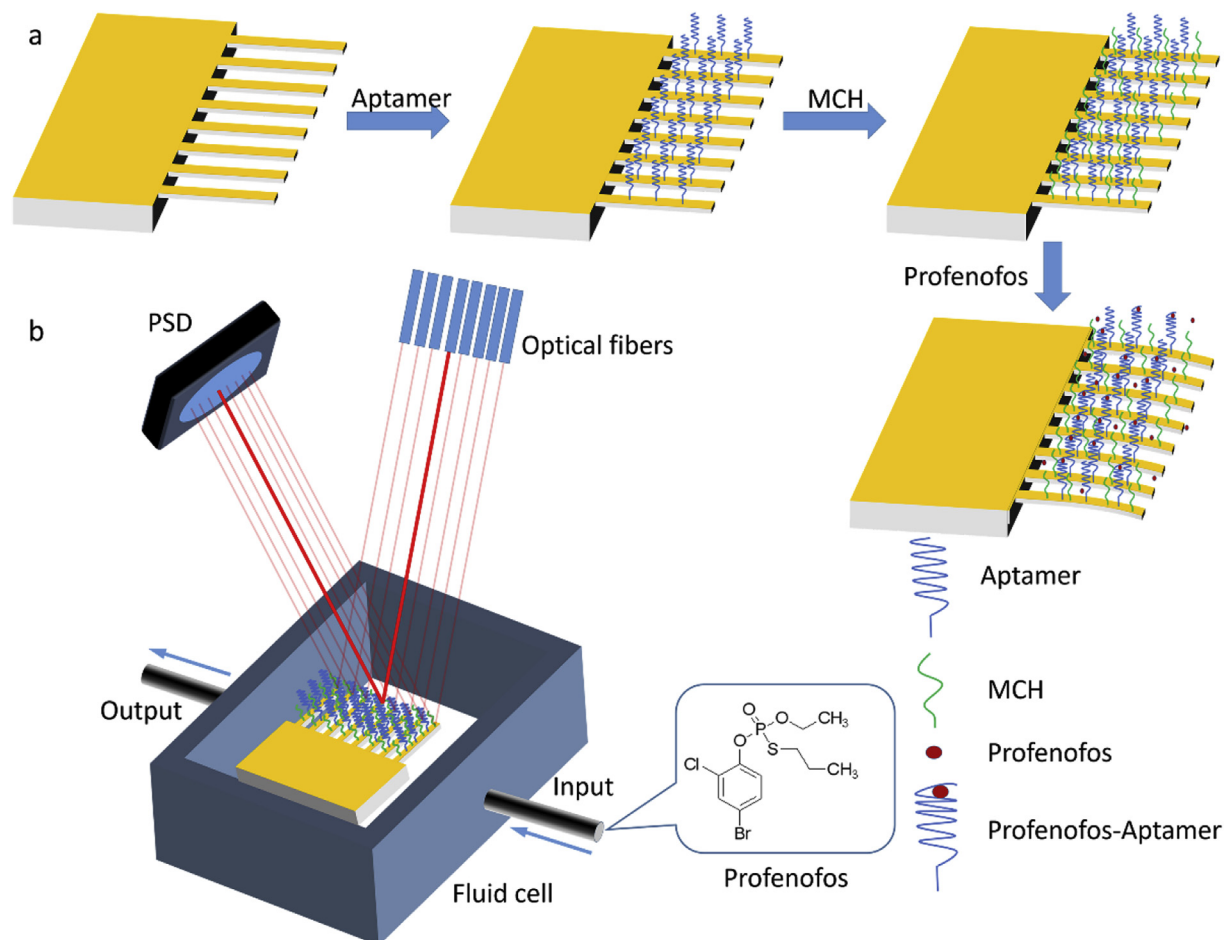


Fig. 1. Schematic diagram of microcantilever modification processes (a) and the microcantilever array instrument based on optical fiber (b). MCH, 6-mercapto-1-hexanol; PSD, position sensitive detector.

2.3. Microcantilever-array functionalization

The aptamer immobilization process was performed in a microplate well, which was replaced by a new one in each step. The array was cleaned with piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 = 3:1$) for 5 min and rinsed four times with deionized water. A gentle stream of nitrogen gas was used for drying the array. The microcantilever array was then immersed in 200 μL of a 1 μM aptamer solution and incubated for 2 h at room temperature. After the incubation period, the array was washed with buffer and dried under nitrogen gas. Finally, 200 μL of 1 mM MCH ethanol solution was used to immerse the array for 1 h to block the microcantilevers. The schematic diagram of functionalization is shown in Fig. 1.

2.4. Deflection measurement of microcantilever-array

The functionalized microcantilever array was mounted in the fluid cell (200 μL), and buffer was injected into the cell at a constant rate of $1 \mu\text{L s}^{-1}$ using an injection pump. The temperature of the fluid cell was controlled at $25 \pm 0.01^\circ\text{C}$ and the room temperature was maintained at $25 \pm 0.1^\circ\text{C}$. A position sensitive detector (PSD) was used to monitor the reflected laser beams to measure the deflection of microcantilever array. After deflection was equilibrated in buffer, profenofos dissolved in buffer was added to the fluid cell. At 65 min after the injection of profenofos solution, buffer was added to the fluid cell. During the detection process, microcantilever deflection was monitored in real time.

2.5. Preparation of vegetable-sample solution

Chinese chives (obtained in a local market) were used as the vegetable sample for detecting profenofos. Chinese chives (20 g) were cut into pieces and immersed in 100 mL of buffer solution. The mixed solution was shocked for 2 min, and let it stand for 30 min. Then we filtered the solution with filter papers (pore size 30, 10 and 5 μm). The filtered solution was used as the sample solution. We added different amounts of $10 \mu\text{g mL}^{-1}$ profenofos solution to the sample solution for detection.

3. Results and discussion

3.1. Detection of profenofos with a microcantilever array

The sequence of the profenofos-specific aptamer (SS2-55) was previously determined [38]. Aptamer SS2-55 is composed of 55 nucleotides, and a $\text{SH}-(\text{CH}_2)_6$ group was attached to the 5' end of SS2-55 in the aptamer synthesis process. Using the thiol group, the aptamer was immobilized on one side of the microcantilever through Au-S chemical conjugation in a single step. During the next modification process, to prevent non-specific adsorption, MCH was used to block the gold side.

For the microcantilever-array sensor, consistency is very important. Under the same conditions, eight identically treated microcantilevers in an array should exhibit similar deflections. To examine array consistency, we measured the deflection of all eight

microcantilevers in the array in response to 200 ng mL^{-1} profenofos. All microcantilevers were modified with aptamers and blocked with MCH. After the deflection was stable in buffer for $>30 \text{ min}$, 200 ng mL^{-1} profenofos solution dissolved in buffer was injected, and after 65 min, buffer was injected into the fluid cell (Fig. 2). Eight microcantilevers in the array had similar deflections ($123.5 \pm 4.6 \text{ nm}$), demonstrating good consistency in the aptamer-based cantilever-array sensor for detecting profenofos.

3.2. Deflection of the microcantilever array in response to different profenofos concentrations

To realize quantitative detection for profenofos, we investigated the relationship between the average deflection of microcantilever array and the concentration of profenofos. After deflections were stable for $>30 \text{ min}$ in buffer, different concentrations of profenofos solution dissolved in buffer were injected, respectively. Every concentration represented a separate experiment using a new microcantilever array. The deflection results for different profenofos concentrations are shown in Fig. 3. The deflection toward the gold side was defined as positive. The average deflection shown in the graph represents the average value of eight microcantilevers in an array. Therefore, the deflection caused by the reaction between the aptamer and profenofos was negative, indicating that the microcantilever bent backward the gold layer, thereby exhibiting compressive surface stress on the gold surface. At 150 min, the average deflection of one array in response to 5, 10, 50, 200, and 1000 ng mL^{-1} profenofos was 46, 71, 104, 130, and 182 nm, respectively. By contrast, when we detected 1000 ng mL^{-1} profenofos using a reference microcantilever array, which was only blocked with MCH and not functionalized with an aptamer, the microcantilever array did not show a significant deflection. Moreover, when detecting another organophosphorus pesticide [ethyl parathion (200 ng mL^{-1})] using the profenofos-specific aptamer-functionalized microcantilever array, the average deflection of the array was far less than that observed for the same profenofos concentration. Our results indicated that the specific binding between the profenofos-specific aptamer and profenofos caused the deflection of microcantilever array. These data revealed that the aptamer-based microcantilever-array sensor showed good specificity for detecting profenofos.

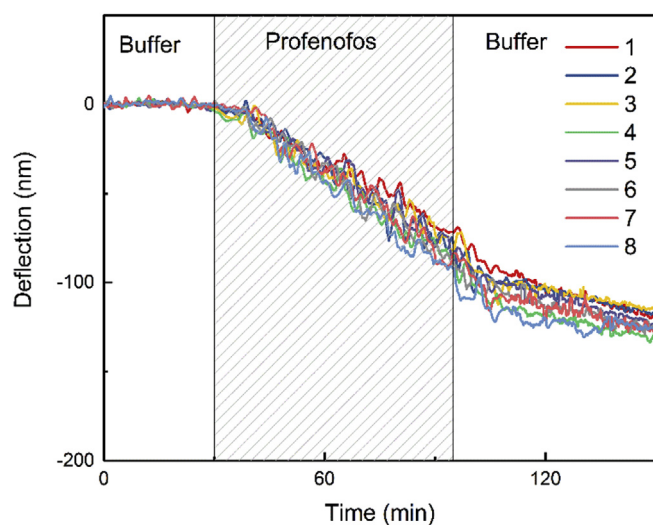


Fig. 2. Real-time deflection of the eight microcantilevers in an array for 200 ng mL^{-1} profenofos. The shaded area represents the injection of the profenofos solution. The numbers in the figure represent the number of the microcantilever in the array.

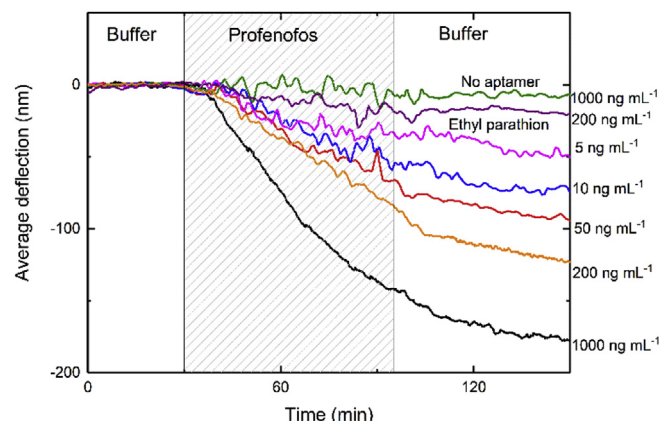


Fig. 3. Real-time average deflection of eight microcantilevers in an array for different concentrations of profenofos ($5, 10, 50, 200$, and 1000 ng mL^{-1}). The shaded area represents the injection of profenofos solution.

The maximum deflection of the array is shown in Fig. 4. Each data point in the graph represents the average deflection of microcantilevers in one array and error bars represent standard deviations. The microcantilever–array deflection showed a positive relationship with profenofos concentration, indicating that the aptamer-based microcantilever was able to quantitatively detect profenofos. The detection limit in our experiments was 1.3 ng mL^{-1} (3.5 nM ; the molecular weight of profenofos is 373 Da), at a signal-to-noise ratio of 3. The results demonstrated that the microcantilever-array sensor based on aptamer showed high sensitivity for profenofos detection.

Many analytical methods have been reported for detecting profenofos using aptamers. A comparison of these aptamer-based methods is shown in Table 1. The detection limit of the microcantilever-array sensor in our experiments (3.5 nM) is 4–4000 times lower than those of other methods. Aptamer-based microcantilever array sensor needs only one step to immobilize the aptamer probes and do not need fluorescent labels or molecular beacons, making it obviously convenient and economical. Moreover, the aptamer-based microcantilever array sensor is capable of achieving high throughput, quantitative and real-time profenofos

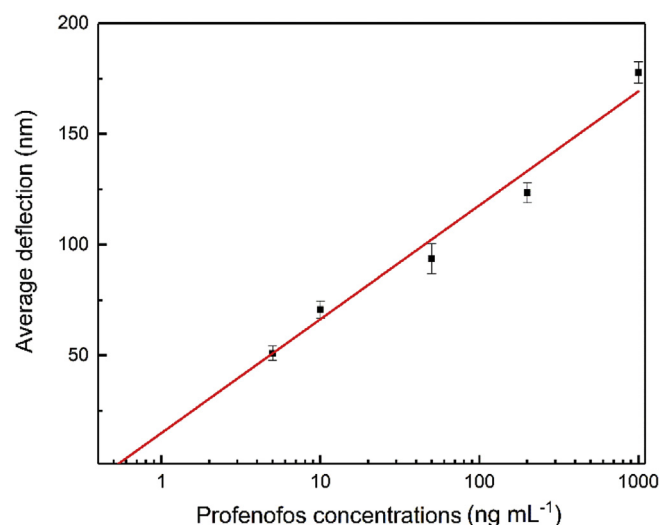


Fig. 4. Maximum average deflection of eight microcantilevers in the array for different concentrations of profenofos solution.

Table 1
Comparison of the profenofos-detection limits of different aptamer-based methods.

Aptamer-based technology	Detection limit	Reference
Fluorescence polarization aptamer array	13.4 nM	[41]
Fluorescence array	14.0 μ M	[46]
Surface enhanced Raman scattering method	14 μ M	[47]
Gold-based nanobeacon probe for fluorescence	0.134 μ M	[39]
Microcantilever-array sensor	3.5 nM	This work

detection. In summary, the combination of aptamer and microcantilever array provides a new method for profenofos detection with the advantages of low-cost, high throughput, simple, sensitive and label-free.

In our experiments, microcantilever arrays were not reused in new experiments, while the reuse of arrays was important for microcantilever array sensor. In previous literature [43,44], the DNA denaturation showed the ability to dissociate target molecules and aptamers, which might be used in the regeneration of functionalized microcantilever array and presented the opportunity for the reuse of microcantilever arrays. Further investigations on the profenofos-specific DNA aptamer denaturation and the reuse of aptamer functionalized microcantilever arrays are required.

A previous report showed that interactions between aptamer SS2-55 and profenofos alter the aptamer structure [41]. When the aptamer interacts with profenofos, the structure of the aptamer changes from a single-stranded molecule to a three-dimensional conformation showing four ring structures, which perform a key role in organophosphorus-pesticides binding. As shown in Fig. 5, when aptamers react with profenofos, the conformation of aptamers will change and the aptamers may occupy additional spaces on the surface of the microcantilever. Therefore, the

repulsion interactions between aptamer molecules may increase. Because the 5' end of the SS2-55 aptamers are immobilized on the gold surface of the microcantilever, these conformational changes may induce repulsive forces on the microcantilever surface and deflect the microcantilever backward the gold surface.

3.3. Detection of profenofos in vegetable-soak solution

Here, we chose Chinese chive as the vegetable sample for profenofos detection. The aptamer-functionalized microcantilever arrays were used to detect different concentrations of profenofos dissolved in the sample solution of Chinese chive, and each concentration represented a separate experiment using a new microcantilever array. The average deflections of eight microcantilevers in an array for different profenofos concentrations in sample solutions are shown in Fig. 6. When the sample solution was injected, the microcantilever array responded immediately, which differed from the deflection measured in the solution of profenofos dissolved in buffer. When buffer was re-injected, the response quickly recovered. In all of the experiments, the activity curves were similar. The retained response following buffer injection should represent the deflection induced by the specific binding between profenofos and aptamer. The average deflections of the array at 150 min for 5 ng mL⁻¹ and 200 ng mL⁻¹ profenofos in the sample solution were 38 nm and 184 nm, respectively, which were close to the deflections measured for the corresponding concentrations of profenofos solution in buffer (Fig. 3). No significant response was observed when the aptamer-functionalized microcantilever array reacted with the Chinese chive sample solution in the absence of profenofos.

Due to the high sensitivity, nonspecific response is an important interference in microcantilever experiment. Compared with the

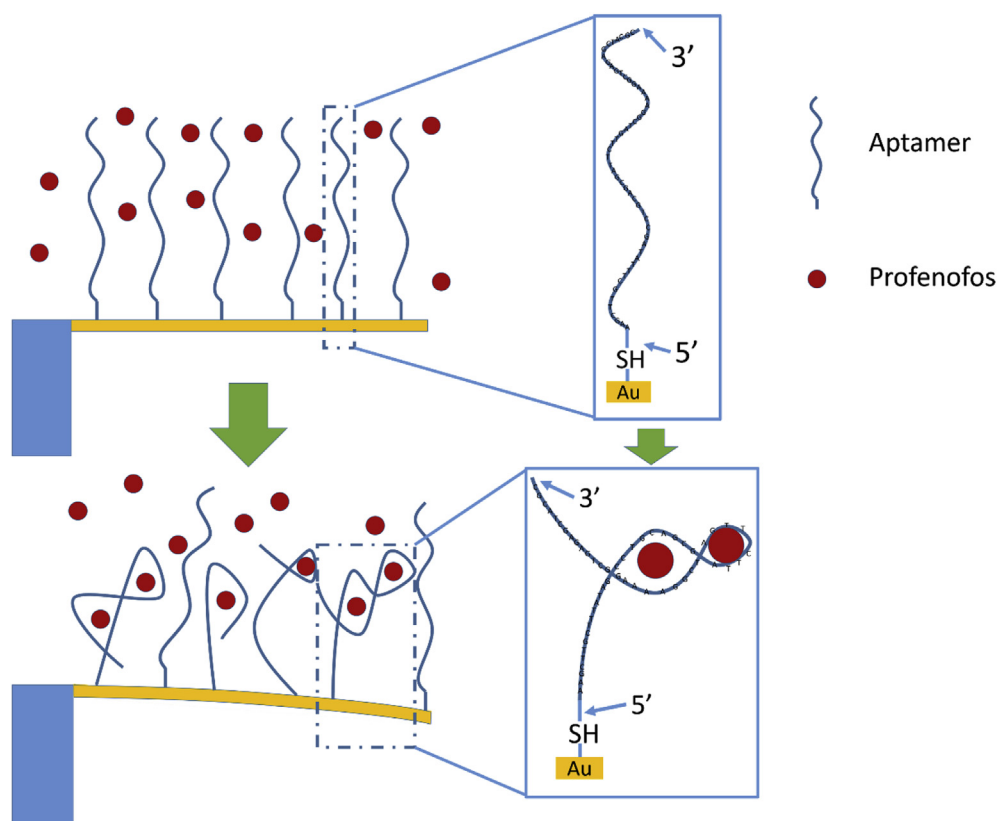


Fig. 5. Schematic diagram of deflection caused by the interactions between the aptamer and profenofos on the surface of microcantilever.

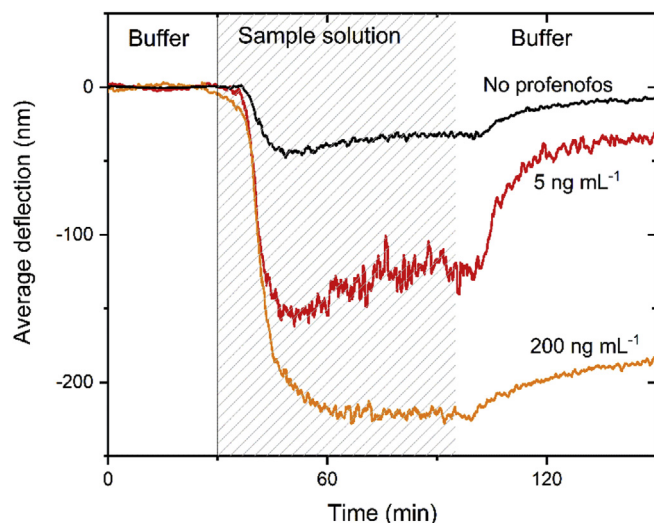


Fig. 6. Real-time average deflection of eight microcantilevers in an array for different concentrations of profenofos dissolved in Chinese chive sample solution.

detection of standard profenofos sample solution (Fig. 3), there was an obvious response in the detection of profenofos with vegetable-soak solution (Fig. 6). To decrease nonspecific factors in the liquid environment, buffer was injected at 95 min to replace the vegetable sample solution in the fluid cell. With the replacement of buffer, the responses decreased rapidly but some signals were well retained, and the retained signals were close to the specific responses shown in Fig. 3. These results indicated that there was obvious nonspecific responses after the injection of vegetable-soak solution and the buffer replacement reduced the nonspecific responses. In our experiment, the vegetable sample solution was only filtered for several times. Therefore, the vegetable sample solution may contain many impurities. It was reported that in microcantilever sensor system based on optical lever read-out method, the changes of solution composition and refractive index could cause nonspecific response [45]. When the vegetable-soak solution flowed into the fluid cell, there might be changes of solution composition and refractive index, which might produce obvious nonspecific responses. The results demonstrated that the aptamer-based microcantilever-array sensor was capable of detecting profenofos in Chinese chive sample solution.

4. Conclusions

In summary, we developed an aptamer-based microcantilever-array sensor for detecting profenofos using a self-developed microcantilever-array instrument based on optical fiber. The aptamer was immobilized on one side of the microcantilever through Au-S chemical conjugation using a one-step immobilization. The specific interaction between profenofos and the aptamer caused a significant deflection of the microcantilever, with the average deflections of the microcantilever arrays showing a positive correlation with profenofos concentrations ranging from 5 ng mL^{-1} to 1000 ng mL^{-1} . These results demonstrated that the microcantilever-array sensor quantitatively detected profenofos at detection limit as low as 1.3 ng mL^{-1} (3.5 nM), which was superior to those of other reported aptamer-based detection methods. Additionally, profenofos dissolved in vegetable-soak solution was detected using the microcantilever-array sensor, showing the ability of this method to detect profenofos in a complex environment. Compared with other aptamer-based methods for detecting profenofos, the microcantilever-array sensor, which constitutes a

label-free, convenient, high-throughput, and rapid method, showed high sensitivity. As recognition molecules, aptamers are low-cost, convenient and easy to be modified, and had an excellent performance in profenofos detection with high affinity, specificity and stability. The aptamer-based microcantilever array sensor showed great potential for pesticide detection in the fields of food security and environmental pollution.

Acknowledgments

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