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Development of Piezoelectric Flow Immunosensor for Competitive Determination of Triazophos

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A piezoelectric immunosensor based on a competitive format was developed for determination of triazophos. Surface modifications *via* three self-assembled monolayers were investigated, respectively and a better result was obtained with the self-assembled monolayers of 3-mercaptopropionic acid. The quartz crystal microbalance based immunosensor was fabricated by immobilizing hapten conjugate (THBu-OVA conjugate) onto the surface of the 3-mercaptopropionic acid-modified electrode and allowing competition between free triazophos and triazophos monoclonal antibodies to occur. The assay exhibited a working range of 5-5000 ng/mL. The triazophos antibody modified quartz crystal cross-reacts with parathion and chlorphrifos.

Key Words: Piezoelectric flow immunosensor, Quartz crystal microbalance, Self-assemble, Pesticide residue.

INTRODUCTION

Increasing awareness about the presence of pesticide residues in the environment has been urging the search for simple detection methods. Classical chromatographic analysis (liquid or gas chromatography) are very sensitive and standardized techniques. Nevertheless they often are too laborious and time-consuming. Furthermore, they need complex and expensive instrumentation often available only in very well equipped and centralized laboratories[1].

Immunosensing is becoming attractive because antibodies can be produced against pesticide molecules with low molecular mass[2-4]. Immunosensors can obtain quantitative results with similar or greater sensitivity, accuracy and precision than other analytical methods because of the availability of high-quality antibodies against target analytes[5]. Piezoelectric quartz crystal based immunosensors have been used to estimate environmental pollutants needs[6,7]. These systems are based upon a variation in the propagation speed of surface acoustic waves (SAW) or bulk waves (BW) of a quartz crystal due to mass changes in the biomolecules bound to the coated layer[8]. The relationship between frequency shift and mass change follows the equation derived by Sauerbrey[9]:

$$\Delta f = \frac{C_f}{A} \Delta m \quad (1)$$

where the measured frequency changes is linearly proportional to the ratio of the mass load (Δm) to the crystal exposed surface (A), C_f is a constant.

Because of their simplicity, low cost and real-time response, piezoelectric quartz crystal sensors are gaining an increasing importance as competitive tools of biomolecular interactions[5]. Self-assembled monolayer (SAM) techniques of depositing functionalized monolayers for the immobilization of biological compounds onto gold surface have been reported in literatures to study piezoelectric immunosensors[10-13]. There are several advantages for using the self-assembled monolayer techniques to develop piezoelectric immunosensor. First, an organized monolayer offers the less resistance for the entrance of analyte to the active sites of the coating material compared to conventional coating using interconnecting channels and hence, it could reduce the response time of the piezoimmunosensor to attain equilibrium. Second, the assembly of self-assembled monolayer is relatively easy and less dependent on special technique and equipment, because self-assembled monolayer is formed spontaneously by the immersion of an appropriate substrate into a solution of an active surfactant in an organic medium. Third, a monolayer of stable and ordered film can be formed through self-assembled monolayer in an ambient environment[14].

In this study, we proposed a simple and sensitive piezoelectric flow immunosensor for rapid detection of triazophos based on immobilization of the hapten conjugate onto 3-Mercaptopropionic acid, O-Mercaptobenzoic acid, L-Cysteine (MPA/MBA/CYS) self-assembled monolayers with 1-ethyl-3-(3-dimethylaminopropyl)carbodimide hydrochloride and N-hydroxy sulfosucinimide as a reactive intermediate. According to the experimental results, optimization and characteristics of the piezoelectric immunosensor were discussed.

EXPERIMENTAL

Triazophos, hapten conjugate (THBu-OVA) and the monoclonal antibodies to triazophos were provided by Institute of Pesticide and Environmental Toxicology of Zhejiang University. 3-Mercaptopropionic acid, O-Mercaptobenzoic acid and L-Cysteine were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Coupling agents, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxy sulfosuccinimide (NHS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Phosphate buffered saline (PBS) (0.01 M, pH 7.4) containing 0.138 M NaCl and 0.0027 M KCl. All chemicals used were of analytical grade or better quality. All solutions were prepared using deionized water from Millipore (Milli-Q, 18.2 MΩ cm, Bedfored).

Fabrication of the piezoelectric immunosensor: The immunosensors were fabricated on 8 MHz AT-cut quartz crystals (diameter 13.7 mm) coated with polished gold electrodes (diameter 5.1 mm, thickness 1000A) on both sides (Chenhua Instrument Manufacturing, Shanghai, China). The crystals were pretreated with 1 M NaOH for 20 min, 1 M HCl for 5 min and Piranha etch solution (1:3 (v/v) 30 %

H_2O_2-98 % H_2SO_4) for 1 min, in sequence, to obtain a clean and highly hydrophobic gold surface. After each pretreatment, the crystals were rinsed with ethanol and water successively and dried with nitrogen[15].

SAM-based immunosensors: The cleaned crystals were incubated with 0.01 M MPA/MBA/CYS (3-Mercaptopropionic acid, O-Mercaptobenzoic acid, L-Cysteine) for 3 h at 37 ºC, respectively. After rinsing with ethanol and water, the MBA/MBA/LYS were treated with 1000 µg/mL EDC-250 µg/mL NHS for 20 min to convert the terminal carboxylic group to an active NHS ester, then, the coupling of THBu-OVA on the gold surface was performed with 20 µL of 1 mg/mL THBu-OVA in phosphate-buffer saline (10 mM, pH 7.4) for 2 h at 37 ºC. After rinsing with ethanol and distilled water, 1 % BSA in PBS was used to block the non-reacted spots of MPA/ /MBA/CYS on the gold surface. After rinsing with PBS and water, the crystals were dried with nitrogen, the sensors were finally ready for further use. The sensors were stored at 4 ºC before use. The preparation procedure and schematic structure are shown in Fig. 1.

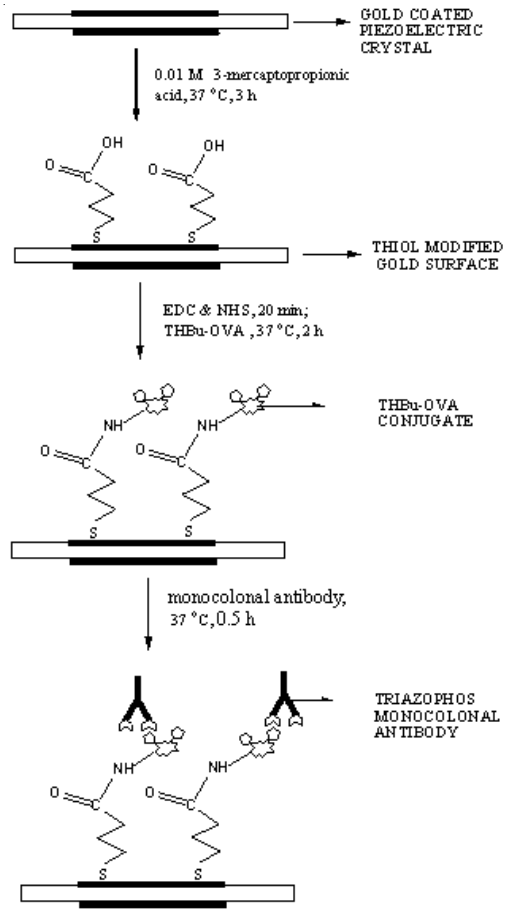


Fig. 1. Schematic diagram for fabrication and pesticide competitive determination

Analytical procedures: A flow cell from the same supplier of the quartz crystals was used to fix the quartz crystal microbalance electrode. The cell was composed of acrylic, with an upper and lower piece held together with two screws. The sensor was sealed between two O-rings in the upper and lower pieces. One face of the sensor was exposed to a 70 μL chamber, which was connected to a Cole-Parmer ten-syringe infusion/withdrawal pump (Cole-Parmer Inc., Shanghai, China) through 0.76 mm ID PVC pump tubing (Gilson Inc., Shanghai, China). At first, phosphate buffer saline was pushed by the infusion pump through the chamber at a flow rate of 0.40 mL/min and the frequency shift was recorded as a function of time. After the baseline obtained with phosphate buffer saline was stabilized, the liquid flow was switched to 0.40 mL/min sample solution of triazophos and antitriazophos. After running sample solution for 0.5 h, the liquid flow was switched back to 0.40 mL/min phosphate buffer saline. The sensor was thus rinsed *in situ* with phosphate buffer saline until obtaining another stable baseline. As shown in Fig. 2, a flow injection system was set up to be used in the triazophos analysis.

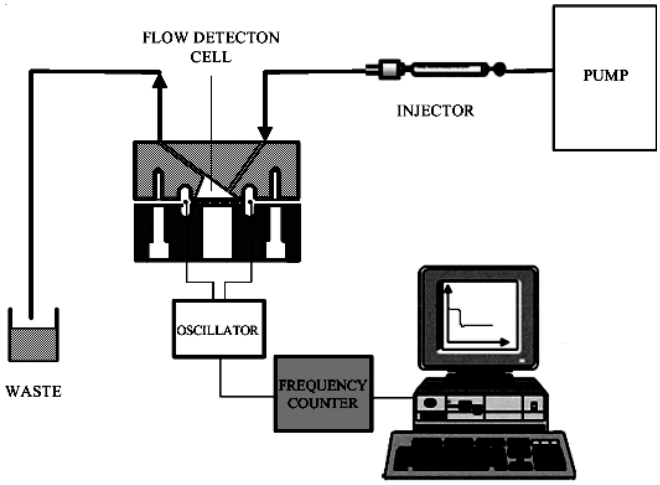


Fig. 2. Flow injection system used in the triazophos analysis

RESULTS AND DISCUSSION

Low molecular weight compounds, such as pesticides, should be measured using competitive assay format. In this way, a higher sensitivity is obtained as mass changes result from binding of the big antibody molecule[16]. As antibody immobilization often leads to impaired regeneration capability and poor immunoassay reproducibility of immunosensors, the conjugate-coated assay format was chosen because of its excellent performance in terms of stability and reliability. Furthermore, covalent binding *via* self-assembled monolayer ensures highly ordered protein immobilization, which provides numerous advantages, *e.g.*, as improvement of detection limits, reproducibility and reusability and prevention of non-specific binding of biomolecules[17].

Different self-assembled monolayers for antigen immobilization: The stepwise assembly of the immunosensor was characterized by quartz crystal microbalance. Both *in situ* and *ex situ* methods were used to record the frequency change. The results are presented in Table-1. In this work, the process of SAM modification, THBu-OVA immobilization, EDC/NHS activation and BSA blocking were monitored in *ex situ* method. The resonant frequency of the same sensor was measured in gas phase before and after immersion.

TABLE-1
DIFFERENTIAL FREQUENCY SHIFT OBTAINED FOR
DIFFERENT SELF-ASSEMBLED MONOLAYERS (n = 5)

Type of SAM	+ THBu-OVA Δf (Hz) ± SD	+ BSA Δf (Hz) ± SD	+ Antitriazophos Δf (Hz) ± SD
MPA	140 ± 6	17 ± 3	126 ± 5
MBA	64 ± 4	44 ± 4	82 ± 4
CYS	75 ± 4	35 ± 4	58 ± 4

We placed the SAM--THBu-OVA--BSA modified crystals in the flow cell. At first, phosphate buffer saline was pushed by infusion pump through the chamber at a flow rate of 0.40 mL/min and the frequency shift was recorded as a function of time. After the baseline obtained with phosphate buffer saline was stabilized, the liquid flow was switched to 0.40 mL/min sample solution of 20 μg/mL triazophos monoclonal antibodies. After running sample solution for 0.5 h, the liquid flow was switched back to 0.40 mL/min phosphate buffer saline. The sensor was thus rinsed *in situ* with phosphate buffer saline until obtaining another stable baseline.

Eva Baldrich[18] reported that antibody incorporated *via* COOH-groups performed better than those conjugated *via* their NH-ter. However, according to Mendes[19] that monolayers possess -NH_2 terminal groups provided the best results for enzyme immobilization. Because no report is available for the biological macromolecule immobilization, we tested three simple SAMs immobilization methods by which the hapten conjugates bound to the QCM electrode. O-Mercaptobenzoic acid and MPA were the SAMs only with -COOH end group and CYS possess -NH_2 terminal groups. A comparison of the three based on SAMs immobilization methods showed that MPA modified sensor to be the best in terms of antigen immobilization and antibody detection, it indicated that the short chain alkyl-thiol SAMs used to create carboxylic-acid-functionized monolayers are suitable for the attachment of protein after activation. 3-Mercaptopropionic acid covers the surface more than MBA and, thus, the number of hapten conjugate binding sites is higher, allowing binding with the antitriazophos more effectively.

Quartz crystal microbalance (QCM) immunoassay optimization: For QCM immunoassay optimization, triazophos was chosen as model analyte. To select suitable concentration of protein-conjugate providing the best assay performance, based on the QCM resonant frequency changed observed during the interaction of 20 μg/mL monoclonal antibody with SAM--THBu-OVA--BSA modified crystals. The four

different THBu-OVA concentration levels were 0.1, 1.0, 10.0 and 20.0 mg/mL. As shown in Table-2, the influence of THBu-OVA concentration on the variation of frequency reached an asymptotic maximum at around 20 mg/mL. To provide reasonable signal with minimum THBu-OVA wasting, a preliminary concentration of 10 mg/mL was chosen for further experiments.

TABLE-2
EFFECT OF THE HAPTEN CONJUGATE (THBu-OVA) CONCENTRATION (n = 5)

THBu-OVA concentration (mg/mL)	Δf (Hz) ± SD (After modified with hapten conjugate)	Δf (Hz) ± SD (detection of 20 μg/mL antitriazophos)
0.1	52 ± 3	9 ± 2
1.0	106 ± 5	74 ± 4
10.0	131 ± 5	118 ± 5
20.0	140 ± 6	126 ± 5

With a 10 mg/mL concentration of THBu-OVA, the method was sensitive enough to determine antibody in the 5-30 μg/mL concentration range. The detection limit varies slightly with different crystals. This may be due to the variation of different crystals modified. Although the exact same procedure was followed, the immobilization may be affected by many factors such as temperature and air pressure changes and non-specific adsorption. As monoclonal antibody concentration increases, the reproducibility of the frequency shift is generally improved. Fig. 3 shows typical responses of MPA--THBu-OVA--BSA modified crystal to monoclonal antibody. The frequency changes leveled off at 20 μg/mL.

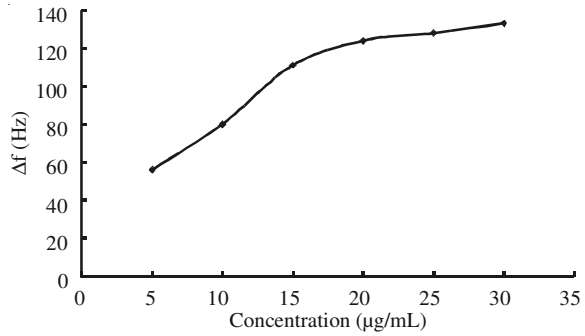


Fig. 3. Frequency decreases at various antitriazophos concentration (n = 5)

In summary, as regards immunoreagents, the optimal conditions chosen were 10 mg/mL of THBu-OVA conjugate for immobilization and 20 μg/mL triazophos monoclonal antibody in phosphate buffer saline.

Reusability: One of the main advantages of immunosensors upon other immunological techniques relies on their ability to be reused as many assay cycles as possible. Therefore, the reliability and stability of an immunosensor greatly depend on its

regeneration capability[1]. A solution of 8 M urea can remove the bound antibody[14] and demonstrated the desorption of the adsorped antibody in present study. The reusability of the THBu-OVA modified crystal was tested. In the flow through system (Fig. 4), the frequency decreases were measured after incubation with triazophos monoclonal antibody (20 µg/mL). The recovered activity was calculated for each restoration. After five regenerations, the activity decreased to 65.5 % for THBu-OVA crystals. Table-3 shows the frequency shifts for each regeneration.

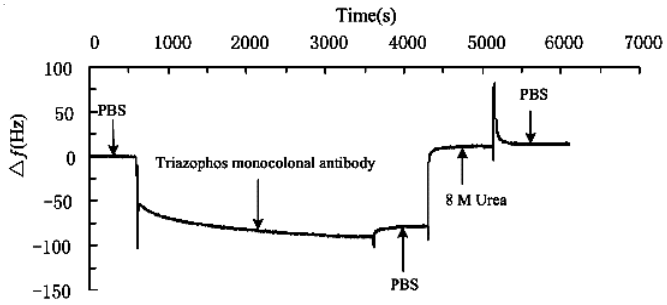


Fig. 4. Frequency response of THBu-OVA modified quartz crystal to the presence of 20 µg/mL triazophos monoclonal antibody, 8 M urea and 0.01 M PBS (pH 7.4)

TABLE-3
FREQUENCY SHIFT AFTER REGENERATION (n = 5)

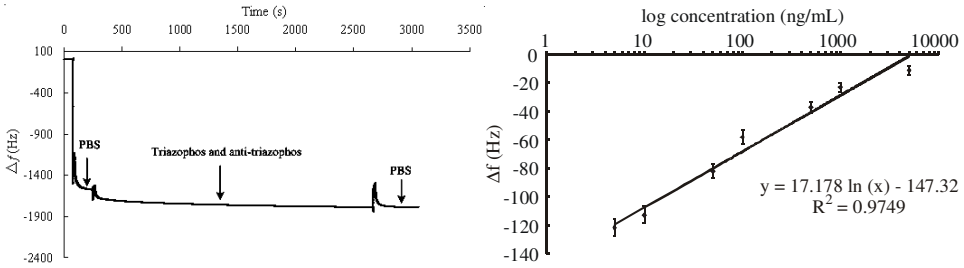
Regeneration No.	Frequency shift (Hz) ± SDE	Recovered activity (%)
1	127 ± 5	100.0
2	116 ± 5	91.3
3	109 ± 5	85.8
4	97 ± 4	76.3
5	85 ± 4	65.5

Competitive analysis of triazophos: The THBu-OVA modified crystal was used for the competitive analysis of triazophos in the flow injection system (Fig 5a). As triazophos is a small molecule, its direct binding would not produce a measurable frequency change when the sample concentration is low. A direct competitive format was used for triazophos detection. After mixing the different concentration of triazophos and 20 µg/mL monoclonal antibody, we extracted 300 µL mixed solution and injected it into the flow system cell, the sample would compete with the antigen conjugate modified on the gold electrode surface for the triazophos monoclonal antibody.

Therefore, the increase in frequency have been observed with frequency shift depending on triazophos concentration (Fig. 5b). The increases in the resonance frequency were due to the competitive loss of monoclonal antibody from the crystal surface, which formed an antibody-triazophos complex in the flowing solution.

Cross-reactivity with various organophosphorus pesticide: The selectivity of the quartz crystal microbalance immunosensor was evaluated by assessing its

response to several analyte-related compounds[1,20]. Cross-reactivity was defined as the percentage ratio between the I_{50} value of the target analyte (triazophos) and the I_{50} value of the cross-reacting compound (parathion and chlorphrifos). As shown in Table-4, none of the triazophos related compounds gave cross-reactivities higher than 2.4 %. These results indicate a very specific assay for triazophos.



(a) (b)

Fig. 5. (a) detection procedure; (b) typical calibration curve for the detection of triazophos at high levels (n = 5)

TABLE-4
CROSS-REACTIVITY WITH VARIOUS ORGANOPHOSPHORUS PESTICIDES (n = 5)

Organophosphorus pesticide	I_{50}[a] (ng/mL)	CR (%)
Triazophos	120	100
Parathion	> 5000	< 2.4
Chlorphrifos	> 5000	< 2.4

a: Pesticide concentration reducing the immunobiosensor maximum response to 50 %.

Conclusion

In this work, we have constructed a self-assembled monolayer based piezoelectric immunosensor for rapid and sensitive detection of triazophos. As a small molecule, triazophos was measured using the competitive assay format. A flow injection system provided a high degree of reproducibility and easy calibration for the detection of triazophos. Surface modifications *via* three self-assembled monolayers (SAMs) were investigated, respectively and a better result was obtained with the self-assembled monolayer of 3-Mercaptopropionic acid. We compared different hapten conjugate concentrations which would affect the result of determination, the results showed that 10 mg/mL was a suitable concentration for conjugate immobilization. The assay exhibited a working range of 5-5000 ng/mL. For practical application of the immunosensor, it is necessary to find more efficient regeneration method.

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