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A non-competitive surface plasmon resonance immunosensor for rapid detection of triazophos residue in environmental and agricultural samples

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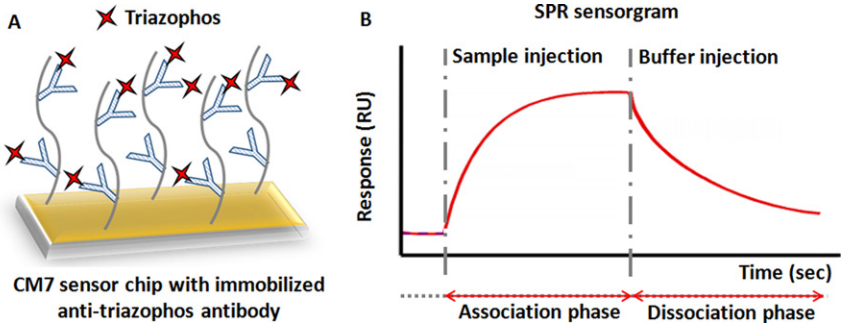
HIGHLIGHTS

- Kinetic analysis of the interaction between triazophos and its monoclonal antibody
- Non-competitive direct SPR immunosensing for triazophos in trace level
- The biosensor method was rapid, reliable and reproducible
- The immunoassay was successfully applied to real samples

GRAPHICAL ABSTRACT



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ABSTRACT

The wide application of an organophosphate pesticide triazophos raises concern on the environmental pollution and the potential risk to human health. Thus, it is crucial to regularly monitor triazophos residue in the environment and agro-products. Herein we described a non-competitive immunoassay for trace detection of triazophos using a direct surface plasmon resonance (SPR) biosensor. Two anti-triazophos monoclonal antibodies (mAbs) were immobilized on the sensor chip and characterized by SPR-based kinetic analysis. The mAb with relatively slow dissociation rate was used for direct immunosensing of triazophos. The biosensor assay showed a high specificity and a low detection limit of 0.096 ng mL^{-1} to triazophos, with the linear detection range of 0.98–8.29 ng mL^{-1}. Under the optimal condition, the sensor chip could be regenerated for 160 cycles at least. Moreover, the sensitive method was applied to determine triazophos in the spiked environmental water and agricultural products, as well as in unknown real-life samples (including Chinese cabbage, cucumber, and apple). Desirable results demonstrated that the newly-developed immunosensor could be used as a rapid, convenient, and reliable tool to regularly monitor triazophos and meet the detection requirement of its maximum residue limits.

1. Introduction

There is a growing concern on the widespread use of pesticides and their possible impacts on public health. Triazophos (*O*, *O*-diethyl-*O*-(1-phenyl-1H-1, 2, 4-triazol-3-yl) phosphorothioate), an alternative to

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highly-toxic organophosphate pesticides, has been extensively used in agriculture practices to control insect pests on cereals, vegetables, fruits, tea, and so on. For example, in China, the annual usage of triazophos was around 10,000 ton in average during the last 5 years. Due to its high chemical and photochemical stability (http://sitem.herts.ac.uk/aeru/iupac/Reports/653.htm), triazophos may remain in the natural environment, enter aquatic ecosystems, and lead to side effects on aquatic biota such as fish (Zhu et al., 2014; Liu et al., 2015a). Recent researches also manifested that triazophos induced oxidative stress and histomorphological changes in rats (Sharma and Sangha, 2014; Sharma et al., 2015; Jain et al., 2011), thereby having potential risk to human health. Maximum residue limits (MRLs) for triazophos in different food products have been announced by many countries, generally ranging from 0.01 to 0.2 mg kg^{-1} (see the details in the Supplementary material). Thus, it is essential to regularly monitor triazophos residue in the environment and agricultural products.

Classic analytical techniques such as high performance liquid chromatography and gas chromatography coupled with different detection modes were maturely used to determine triazophos residue in various samples (Fu et al., 2009; Zhao et al., 2014; Andrade et al., 2015; Hayward et al., 2015). These methods are accurate and reliable, but require skilled technicians, sophisticated instrumentation, high consumption of organic solvent, and complicated sample pretreatment. Acetylcholinesterase-based biosensors for triazophos (Ju et al., 2015; Du et al., 2007) are rapid, easy-to-use, but lack high specificity, as the acetylcholinesterase can be inhibited by both organophosphate and carbamate pesticides. As a popular screening methodology, immunodiagnostics of different formats, such as enzyme-linked immunosorbent assay (ELISA) (Liang et al., 2007; Gui et al., 2010; Gui et al., 2006; Jin et al., 2008), microbead-based immunoassay (Du et al., 2015; Liang et al., 2013; Guo et al., 2013), chemiluminescent enzyme immunoassay (CLEIA) (Jin et al., 2012; Chen et al., 2015), gold immunochromatographic strip test (Guo et al., 2009; Gui et al., 2008), and fluorescence polarization immunoassay (Liu et al., 2016a) have been successfully developed for rapid detection of triazophos residue in food and environmental samples, with the detection limits lower than 0.01 mg L^{-1}. However, those immunoassays are based on the competitive indirect detection mode and the reaction signal is inversely proportional to the amount of analyte, which differs from the direct sandwich mode for large molecules and may be misjudged by inexperienced people. A method that can directly determine the target itself (instead of the unbound antibody from an inhibition reaction) will be more attractive in terms of accelerated procedure, reduced use of bioreagents, and improved confidence of the assay. Till now, only a non-competitive rapid piezoelectric immunosensor for direct determination of triazophos was reported (Huang et al., 2010), but the detection limit (0.04 mg L^{-1}) and working range (0.1–100 mg L^{-1}) could hardly reach the MRL requirement. Other burgeoning immunosensors that have potential for direct measurement were not exploited for trace detection of triazophos.

Surface plasmon resonance (SPR) technology is an optical detection platform that offers real-time and label-free analysis of molecular interaction. In the past two decades, SPR-based immunosensors have been widely applied to detect large molecules, where the analyte's high mass and the use of sandwich immunoassay format can lead to high signal and thus desirable sensitivity (Scarano et al., 2010; Riedel et al., 2014). In contrast, low-molecular-weight (LMW, <1 kDa) compounds present in trace level are very difficult to be directly determined by traditional SPR immunosensors, owing to the small change in the refractive index induced by the binding of those analytes on the sensor surface (Gouzy et al., 2009). Therefore, competitive inhibition SPR immunoassays were often developed for trace analysis of pesticides. That is, a pesticide-protein conjugate served as the competitor was immobilized on the sensor chip and the solution competition occurred when injecting the mixture of antibody and analyte (Hirakawa et al., 2015a; Mauriz et al., 2012; Estevez et al., 2012; Hirakawa et al., 2015b); otherwise, the antibody was immobilized on the sensor chip and the surface competition happened when injecting the mixture of competitor and analyte (Mitchell, 2010). In recent years, other strategies to improve the sensitivity of direct SPR non-competitive immunoassay for pesticides have been explored by employing nanoparticle lables as signal enhancement reagents (Liu et al., 2014; Liu et al., 2015b). Although these methods can meet the requirement for trace detection of pesticides, they need longer assay time, more reagents and complex labeling process. Still, direct SPR non-competitive immunosensors providing more rapid and straightforward diagnostics deserve further investigations for detection of small molecules, despite more challenges.

Recent improvements in SPR instrumentation including lower noise valves and advanced microfluidics with stronger vacuum pumps, have decreased the total noise in these systems and hence resulted in better sensitivity and reliability. However, attempts for trace analysis of LMW analytes using direct SPR non-competitive immunoassays were limited to very few targets (Yakes et al., 2014; Munoz et al., 2011). In this work, we first characterized two monoclonal antibodies (mAbs) against triazophos by ELISA and SPR-based kinetic analysis. After comparison, the optimal mAb was used to develop a non-competitive SPR immunosensor for direct monitoring of triazophos residue in environmental and agricultural samples. The key factors in the assay design and the effects of sample matrices on the assay performance were also discussed.

2. Materials and methods

2.1. Reagents and instrumentation

Triazophos, parathion, chlorpyrifos and other related pesticide standards were obtained from Agro-Environmental Protection Institute, Ministry of Agriculture (Tianjin, China). Each pesticide stock solution with a concentration of 1 mg mL^{-1} was prepared in dimethylsulfoxide (DMSO) and stored at 4 °C. Sensor chip (CM7), phosphate buffered saline (PBS, 0.01 M, pH 7.4) containing 0.05% (v/v) polysorbate surfactant P20 (PBS-P+), the amine coupling kit containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine-HCl (pH 8.5), 10 mM sodium acetate (pH 4.5, 5.0, and 5.5), immobilization and regeneration buffers were procured from GE Healthcare Bioscience AB (Upsala, Sweden). All buffer solutions were prepared with ultra-pure water acquired from a Milli-Q purification system (Millipore, Bedford, USA) and were passed through the membrane filter (pore size, 0.22 μm, Millipore) prior to use. All SPR tests were carried out at 25 °C using Biacore T200 biosensor system (GE Healthcare, Madison, USA). The data acquisition software was Biacore T200 Evaluation Software Version 3.0.

2.2. Production of mAbs against triazophos

Two kinds of mAbs specific to triazophos were previously developed in our laboratory, named as 4B2 (Jin et al., 2008) and 8C10 (Liu et al., 2016b). The mAbs were purified and dissolved in 10 mM phosphate-buffered saline (PBS) to a final concentration of 1 mg mL^{-1}. To make sure the protein activity, titers of mAbs were measured by non-competitive indirect ELISA (iELISA) and the sensitivity to triazophos (IC_{50}, 50% inhibitory concentration) was determined by competitive iELISA. The conjugate of triazophos hapten-ovalbumin (THBu-OVA) (Gui et al., 2006) was used as the coating detective antigen. Standard competition curves were acquired by plotting the inhibition rate against the logarithm of analyte concentration. The ELISA procedures were described in our previous work (Liu et al., 2016b).

2.3. Sensor immunochip preparation

A sensor chip CM7 coated with a high density of carboxymethylated dextran was put into Biacore T200 system. Initially, a solution of 70% (w/

w) glycerol was used for the calibration of flow cell channels (Zhu et al., 2014; Liu et al., 2015a; Sharma and Sangha, 2014; Sharma et al., 2015), so as to normalize the resonance signal in all channels to compensate for small differences. The chip surface was then washed with the running buffer (PBS-P+) at a flow rate of 10 μL min^{-1} for 200 s.

In the preliminary test, 10 mM sodium acetate solutions with different pH values (4.5, 5.0, and 5.5) were respectively used to dilute the anti-triazophos mAbs. It was found that the pre-concentration of the ligand molecules to the sensor surface was highest when the buffer at pH 5.0 was used for dilution. Thus, 10 mM sodium acetate buffer at pH 5.0 was chosen as the dilution buffer for the mAb immobilization.

Each mAb was covalently immobilized onto the sensor chip via amine coupling chemistry. In detail, a 400 μL mixture of 0.1 M NHS and 0.4 M EDC (1:1, v/v) were injected into flow cell channels 1–4 for 1200 s to produce active succinimidyl ester groups on the chip surface. Channels 1 and 3 served as references were then deactivated, while the active channels 2 and 4 were respectively conjugated with the two anti-triazophos mAbs (50 μg mL^{-1}) in 10 mM sodium acetate buffer (pH 5.0) at a flow rate of 10 μL min^{-1} for 900 s to reach the saturation plateau. Afterwards, the unreacted ester groups were deactivated and blocked with 1 M ethanolamine (pH 8.5) for 400 s, resulting in the antibody immobilization level for the active channels 2 and 4 of 30,000–35,000 resonance units (RU). The raw sensorgrams for the whole immobilization procedure were presented in Fig. S1. After being rinsed with the running buffer (10 μL min^{-1}) for 300 s, the sensor immunochip was ready to be used for SPR assays.

2.4. Antibody kinetics/affinity measurement

Immunoaffinity of the anti-triazophos mAb was measured by direct SPR assays using the mode of multiple cycle kinetics. A series of triazophos solutions at different concentrations (0, 1, 2, 4, 8, 16, 32 ng mL^{-1}) were made by the running buffer with the equal content of 5% DMSO. The solutions were injected at the flow rate of 30 μL min^{-1} for 120 s, followed by dissociation for 600 s. After each binding reaction, the mAb-immobilized surface was regenerated with the injection of 10 mM glycine-HCl (pH 2.0) at the flow rate of 30 μL min^{-1} for 30 s. Meanwhile, solvent correction was also performed by injecting 8 solutions of the running buffer (PBS-P+) containing 4.5%–5.8% DMSO, and it was repeated after 20 sample cycles.

The flow paths were set in pairs as 2-1 and 4-3 with minimized dead volume to support accurate normalization. That is, the responses acquired from the detection channels 2 and 4 were normalized by respectively subtracting the signal generated from the reference channels 1 and 3, in order to remove the signal from non-specific binding and the bulk refractive index changes induced from the buffer or sample (Zhu et al., 2015). To correct for DMSO effects and small errors in preparation of the samples, solvent correction was applied before evaluation proceeding. The corrected response (reference subtracted and solvent corrected) from active surface versus cycle number was obtained. For result evaluation, the response of blank control (zero concentration) was also deducted from that of each sample containing triazophos, and the blank-deducted sensorgrams were subjected to kinetic fitting by the Biacore Evaluation Software with a 1:1 binding model.

2.5. Establishment of calibration curves

Calibration curves were derived from the sensorgrams of triazophos standard solutions at gradient concentrations ranging from 0.06–64 ng mL^{-1}, also taken zero concentration as the blank control. Sample solutions were injected at the flow rate of 30 μL min^{-1} for 180 s (longer than that in kinetics analysis), followed by dissociation for 120 s and regeneration for 30 s as described above. Using the end-point data, the sample response was acquired by the average RU value from 10 to 15 s (report point of the stability) after the end of sample injection, to avoid the bulk refractive index contribution from the sample. The corrected and blank-deducted response was plotted against triazophos concentration to yield a standard sigmoidal curve of the SPR immunoassay, using the four-parameter logistic function in OriginPro 8.5.

2.6. Cross-reaction test of the immunosensor

The cross-reactivity of the immunosensor was validated by using other commonly used insecticides, i.e., typical representatives of organophosphates (parathion, chlorpyrifos), pyrethroids (fenpropathrin, deltamethrin, bifenthrin) and neonicotinoids (imidacloprid, acetamiprid, thiamethoxam). All the selected pesticides were individually tested at the same concentration of 32 ng mL^{-1}, as the sensor signal is relevant to the total mass of the bound analyte.

2.7. Stability test of the immunochip

For stability test, reusability of the mAb-based immunochip was evaluated by repeated injection of 32 ng mL^{-1} trizophos solution at interval of 25 cycles per day to monitor the SPR response. During those days, the sensor chip stayed at 25 °C and under running buffer conditions for standby.

2.8. Spiked recovery test

Surface water (Yuhang Tang River, Hangzhou, China) and ground water (Hupao Spring, Hangzhou, China) were collected as the environmental samples. Agricultural products such as Chinese cabbage, cucumber, and apple were purchased from Wal-Mart supermarket (Hangzhou, China). They were first confirmed by gas chromatography–mass spectrometry (GC–MS) to be triazophos-free and were used for matrix effect and spiked recovery determination in the SPR tests.

Each filtered water sample (10 mL) was individually spiked with a few volume (10–50 μL) of triazophos concentrated solution (10 μg mL^{-1} in DMSO) to obtain the final triazophos at 10 or 50 ng mL^{-1}. Aliquots of the spiked water were diluted with 5% DMSO-PBS-P+ at 10-fold (1:10, v/v) and directly injected to the SPR immunosensor.

The homogenized cabbage (10 g), cucumber (10 g), and apple (10 g) samples were spiked with triazophos standard to different levels (10–100 ng g^{-1}). After incubation 2 h at room temperature, they were extracted and purified by QuEChERS (Quick, Easy, Cheap, Rugged, Effective, and Safe) method. The procedures were followed as described in the literature (Du et al., 2015) with some modification (details provided in the Supplementary material).

Each kind of blank control sample pretreatment without the fortification of triazophos was also pursued with the same pretreatment as the spiked one. The blank samples were used to evaluate the non-specific binding to the sensor surface and the interference from matrix components on the binding between triazophos and the mAb attached on the sensor chip.

2.9. Application to unknown real-life samples

To evaluate the practical application of the new immunosensor assay, some unknown real-life samples (including Chinese cabbage, cucumber, and apple) collected from six different local farmers' markets (Hangzhou, China) were analyzed using the proposed SPR assay, followed by GC–MS for the confirmation.

Sample preparation for GC–MS analysis and the instrumental conditions was provided in the Supplementary material.

3. Results

3.1. Characterization of mAbs by ELISA

The development of ultra-sensitive immunosensors requires the use of antibodies with good quality, and usually mAbs are preferable due to

the high specificity and affinity. In this study, two anti-triazophos mAbs were first characterized by iELISAs under the same conditions, with the coating antigen of THBu-OVA at 2 μg mL^{-1}. The titer was determined as the mAb dilution offering the absorbance at 490 nm close to 1.0 in the non-competitive iELISA, and thus the mAb's working concentration was selected for the competitive ELISA, which was used to assess the assay sensitivity to triazophos. Results shown in Table 1 indicated that both 8C10 and 4B2 mAbs gave high affinity to the coating antigen with the titer $>1 \times 10^4$, and the mAb-based competitive iELISAs displayed satisfying sensitivity to triazophos with IC_{50} around 1 ng mL^{-1}. Since ELISAs only provide the end-point data of immuoreactions under equilibrium conditions, more kinetics information should be acquired from other real-time analytical techniques such as SPR biosensors.

3.2. Kinetic evaluation by SPR immunosensor

Ideally, kinetic profiling of hapten-specific antibody should be accomplished by direct analysis of the interaction between the antibody and the LMW analyte itself, rather than the hapten-protein conjugate used in previous studies (Sanders et al., 2016; Hu et al., 2015). In this work, SPR immunosensor for direct detection of triazophos was fabricated by the immobilization of anti-triazophos mAbs to a sensor chip CM7 with high density of conjugation amount, followed by direct monitoring the interaction between triazophos in the solution and the mAb located on the chip surface (Fig. 1).

For kinetic evaluation, standard solutions of triazophos at different concentrations were tested and their final SPR sensorgrams were modeled (Fig. 2). The colored experimental curves generally matched well with the black fitted curves. Table 2 lists the binding kinetic values of the two mAbs against triazophos. Both the association (k_a) and dissociation (k_d) rates were of low standard errors, and the values of Chi^2 indicated the low average squared residuals. Quality control of the two sensorgrams showed that kinetic constants appeared to be uniquely determined, and no significant bulk contributions were found.

Concerning the dissociation constant (K_D) calculated as the ratio of k_d/k_a, both mAbs exhibited strong affinity (10^{-7} to 10^{-10} M for a mAb) to the target and their apparent binding capability looked similar. These findings could be used to explain the results from ELISAs that the two mAbs gave close reactivity to the coating antigen and free triazophos. By comparison of the kinetic parameters (k_a, k_d), the mAb 8C10 bound the target faster than 4B2, while the complex of mAb 4B2 with triazophos dissociated slower than that of 8C10. For concentration analysis, the binding stability is highly relevant to k_d value, the smaller the more stable. Therefore, the mAb 4B2 with the lower dissociation rate was chosen for next experiments.

3.3. Direct SPR immunoassay

3.3.1. Assay selectivity

The selectivity of the mAb 4B2-based direct SPR immunoassay was evaluated by performing cross-reactivity studies to other commonly used pesticides. As shown in Fig. 3, other 8 tested pesticides gave negligible responses (<0.2 RU) when they were injected at 32 ng mL^{-1}, suggesting that the newly-developed immunosensor assay was very specific to triazophos. The good selectivity could be mainly ascribed to the high specificity of the mAb, coupled with the use of the running buffer containing 0.05% polysorbate surfactant to minimize the non-specific binding.

3.3.2. Assay sensitivity

For concentration analysis by direct binding assays, calibration curves were established by detecting a series of triazophos solutions with known concentrations (0.06–64 ng mL^{-1}). The corrected and blank-deducted response at the report point of binding stability was recorded and plotted against triazophos concentration. Fig. 4 shows an average calibration curve of triazophos detected by the mAb 4B2-based immunosensor. Small error bars for the data points indicated a good repeatability of the assay. With the fitted four-parameter logistic equation, the linear range (EC_{20}–EC_{80}) was determined as 0.98–8.29 ng mL^{-1}. The limit of detection (LOD) was calculated to be 0.096 ng mL^{-1}, using S/N = 3 where the noise level is the standard deviation of 6 replicate measurements at zero concentration (Munoz et al., 2011; Zhu et al., 2015).

3.3.3. Immunochip stability

The stability test showed that the mAb 4B2 was fairly tolerant to the regeneration conditions (10 mM glycine-HCl, pH 2.0) that totally liberate triazophos from the surface, with the chip immobilization signal stable at around 32,000 RU. As shown in Fig. 5, the SPR response of trizophos at 32 ng mL^{-1} remained stable until 125 regeneration cycles with its descent rate <2.5%. Afterward, the immunochip was not totally deactivated but the SPR response was weaker, suggesting that some degree of deactivation occurred along with the time used. Taken the cut-off of the descent rate as 10%, the mAb 4B2-immobilized chip could maintain effective for 160 regeneration cycles within a period of 7 days.

3.4. Matrix interference

Different sample matrices lead to different interferences on the assay performance. Ideally, separate calibration curves should be set up for each sample matrix. However, it is not practical to carry out for screening various kinds of samples by rapid methods like immunoassays. Instead, standard curves made from matrix-free buffer solutions are often used in different kinds of immunoassays, in conditions that matrix interference can be reduced by a large dilution of samples or purification methods (Zhao et al., 2015).

In our preliminary tests, matrix effects were evaluated by comparing a matrix-free standard curve with calibration curves obtained in the sample matrices with different dilution times. The curves could be very close when the dilution of sample extract was enough to eliminate the matrix influence. At first, agricultural samples were rapidly extracted by ethyl acetate without purification, and ultra-high dilution (100-fold for Chinese cabbage and cucumber, 200-fold for apple) with the running buffer was required to ignore the matrix interference, but it severely reduced the assay's detectability for complicated samples. A user-friendly method of QuEChERS was then employed for sample pretreatment, with efficiency for cleaning-up complex samples (Zhao et al., 2015). Therefore, much less dilution time (10-fold for Chinese cabbage

Table 1
Characterization of mAbs by iELISAs.[a]

Clone name	Titer ($\times 10^4$)	Working concentration (ng mL^{-1})	Standard equation parameters[b]			IC_{50} (ng mL^{-1})	IC_{20}–IC_{80} (ng mL^{-1})
			Slope	Intercept	R^2		
4B2	24	40.6	16.97 ± 1.14	48.10 ± 5.07	0.9933	1.12 ± 0.11	0.19–6.55
8C10	12	37.8	15.10 ± 1.26	51.55 ± 4.94	0.9918	0.90 ± 0.08	0.12–6.57

[a] Non-competitive or competitive iELISA was performed by coating 2 μg mL^{-1} of THBu-OVA.
[b] The standard equation was defined as y = aln(x) + b (obtained from Microsoft Office Excel 2010, using linear regression model), and data were based on the mean values of three replicates.

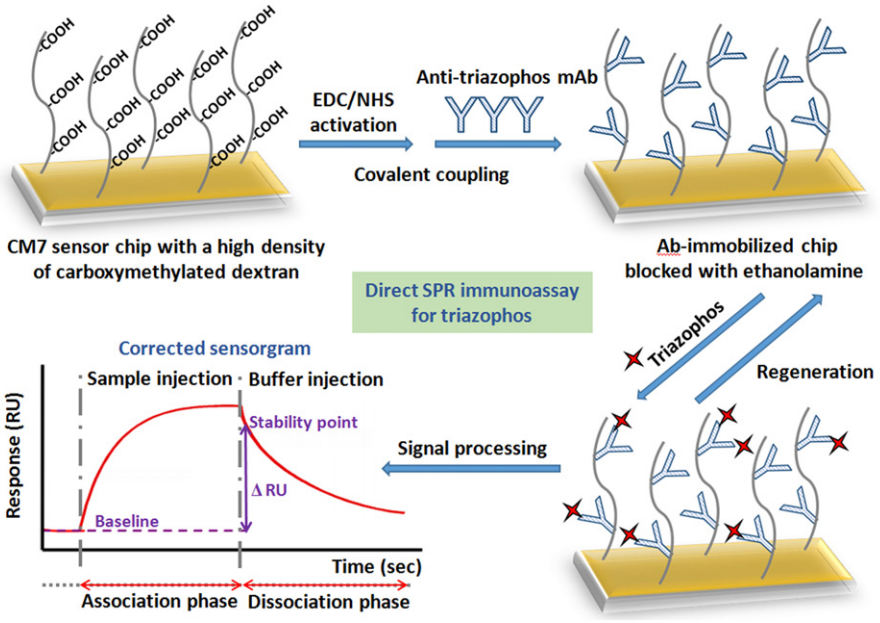

Fig. 1. Preparation of mAb-immobilized sensor chip for direct detection of triazophos and real-time surface plasmon resonance (SPR) sensorgram for association and dissociation of the immunocomplex. The response for the mAb-coated channel was normalized by subtracting the signal from the reference channel, followed by solvent correction and blank deducting.

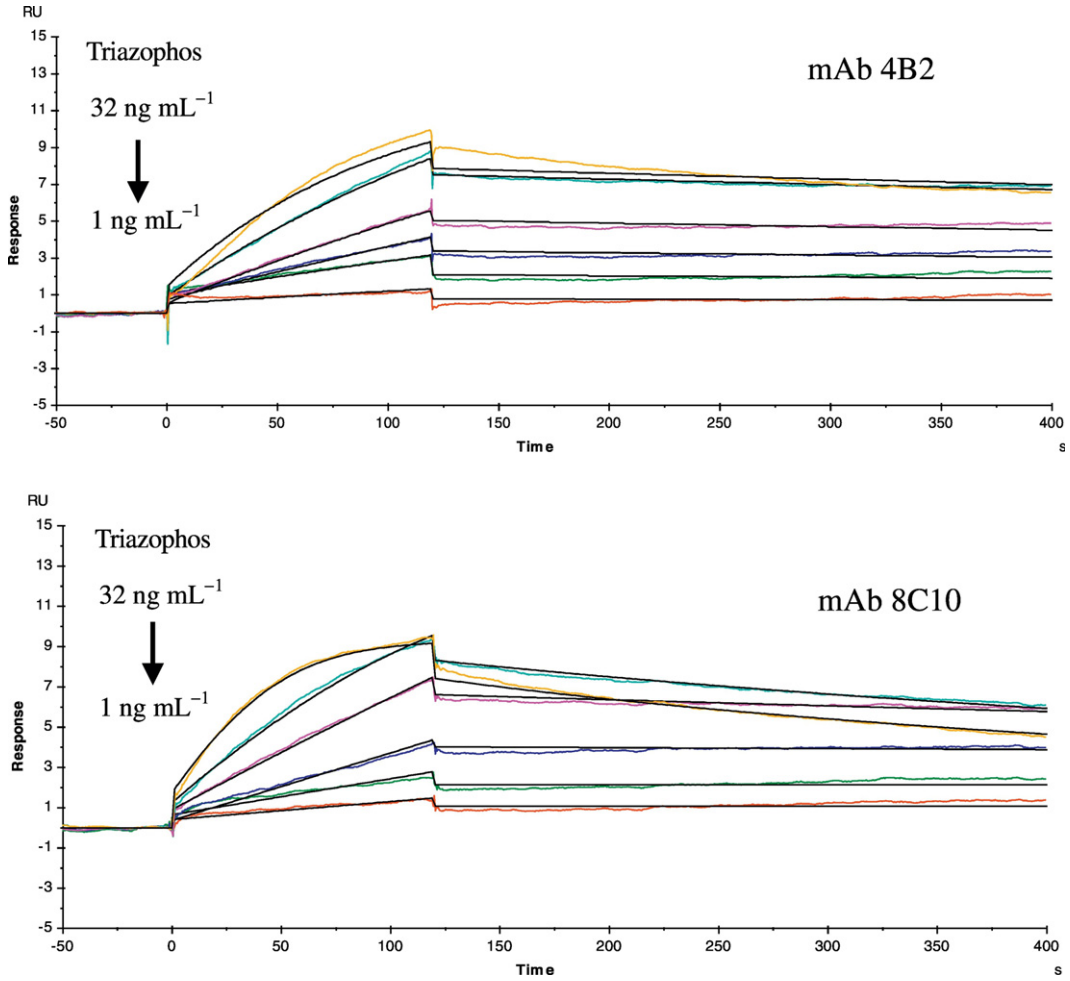

Fig. 2. SPR kinetic sensorgrams (colored lines) and fitting curves (black lines) for the two mAbs' binding to triazophos standard at different concentrations (from up to down: 32, 16, 8, 4, 2, 1 ng mL^{-1}). The responses were reference-subtracted, solvent-corrected, and blank-deducted. The sensorgrams were fit with a 1:1 binding kinetic model.

Table 2
Binding kinetic characterization of the two mAbs to triazophos.

mAb	k_a (1/Ms)	k_d (1/s)	K_D (M)	Chi^2 (RU^2)
4B2	$1.07 \times 10^5 \pm 5.0 \times 10^2$	$4.30 \times 10^{-4} \pm 2.2 \times 10^{-6}$	4.04×10^{-9}	0.098
8C10	$4.24 \times 10^5 \pm 4.6 \times 10^3$	$2.17 \times 10^{-3} \pm 8.7 \times 10^{-6}$	5.12×10^{-9}	0.051

and cucumber, 20-fold for apple) was needed for the purified sample extract to minimize the matrix effects on the SPR tests.

3.5. Recovery tests

Table 3 listed the recoveries of triazophos in spiked samples analyzed by the mAb 4B2-immobilized SPR immunosensor. For environmental water samples, a simple dilution (10-fold) with the running buffer was useful to eliminate the matrix influence, and the mean recoveries of spiked water samples ranged from 90% to 108%. For spiked agricultural samples, good recoveries of 84%–109% were obtained, combining QuEChERS method and a few dilution for preteatment. Coefficients of variation were all below 12.5%, indicating an acceptable precision. These results demonstrate that the developed SPR immunoassay can generally meet the detection requirement of trizophos MRLs in food mentioned-above.

3.6. Analysis of unknown real-life samples

Table 4 shows the results of triazophos residue in some unknown real-life food samples detected by both the proposed SPR assay and GC–MS method. 7 out of 18 samples were found to be triazophos positive, ranging from 11.8 to 64.7 ng g^{-1} by the immunosensor tests. By comparison, the results from GC–MS were largely consistent with those from SPR, where the positive data were from 11.3 to 67.6 ng g^{-1}. The good correlation further demonstrated the reliability of the newly-developed SPR immunosensor.

4. Discussion

It is very difficult to establish non-competitive immunoassays for direct detection of pesticides via the common techniques, as pesticides are LMW compounds that only have single epitopes and the usual sandwich formats can hardly be explored. Likewise, direct SPR immunosensing for small molecules has challenges not encountered with large molecules, because the SPR signal is contributed by the mass changes of binding molecules (Mitchell, 2010). Advanced SPR biosensor has decreased the total noise in the detection system, so that the high signal-to-noise ratios of the measurements can be confidently achieved when monitoring LMW analytes. Moreover, the use of the CM7 sensor chip with high conjugation capacity is favorable to maximize the binding signal for small molecules even at trace concentrations (Yakes et al., 2014). By combining these improvements, direct SPR analysis of trace pesticides such as triazophos, is promising as demonstrated by the work herein.

The primary factor that determines the quality of SPR biosensor data is the quality of the recognition element itself and how it is immobilized on the sensor surface. For direct binding assay of SPR, antibodies are the only bio-reagents needed and their effective immobilization on a sensing platform is very crucial for the sensor sensitivity. In this study, two specific mAbs (4B2 and 8C10) with high sensitivity to triazophos were used for immobilization, as they proved to be very effective in our previously reported immunoassays (Jin et al., 2008). In order to gain sufficient high response for capturing triazophos, activation of the carboxylic acid groups on the chip surface was extended to 20 min and the mAbs were immobilized for enough time to reach the saturation plateau at high level above 30,000 RU. Additionally, the long time of bioconjugation chemistry used to immobilize mAbs with high density on the surface followed by blocking with ethanolamine, can generally decrease the charge density on the carboxymethylated dextran and help to reduce the tendency for non-specific electrostatic binding of analyte to the dextran surface (*Biacore Concentration Analysis Handbook BR-1005-12 Edition AB. In.*, n.d.). Although large amounts of the immobilized mAbs recommended for concentration measurements may promote rebinding cases or limit mass transport and thereby influence the kinetic evaluation, a high flow rate of 30 μL min^{-1} was used in the present work to reduce the possibility of target rebinding or mass transport limitation (Chang et al., 2014). Furthermore, the stability test of the mAb 4B2-based sensor chip indicated its good reusability for 160 regeneration cycles at least, which offered the reliability in concentration analysis. Nonetheless, the reactivity duration of the immunosensor chip was not yet monitored at the shelf-storage condition such as 4 °C.

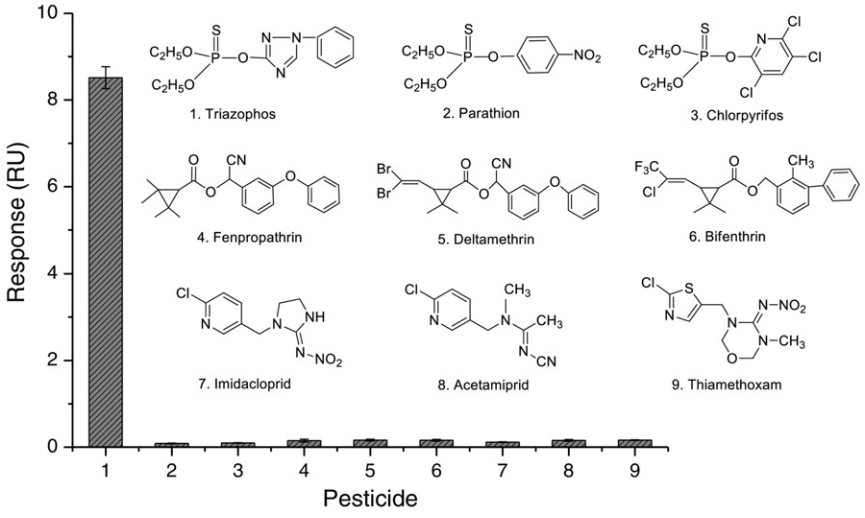

Fig. 3. Selectivity of the mAb 4B2-based SPR immunosensor towards different pesticides at each concentration of 32 ng mL^{-1} (n = 3). The responses were reference-subtracted, solvent-corrected, and blank-deducted.

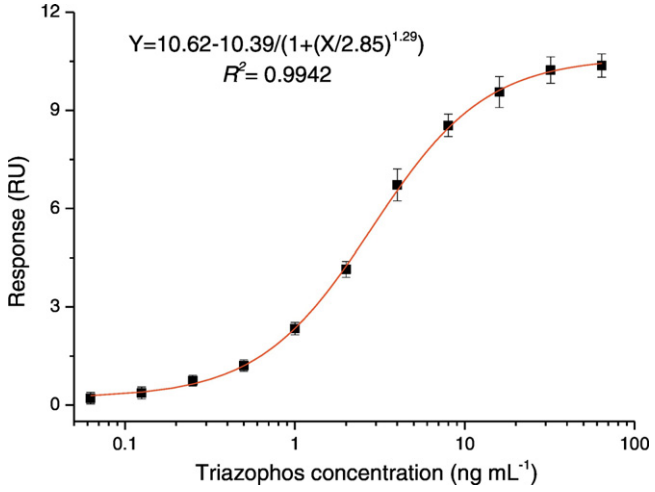

Fig. 4. Standard curve of triazophos by the mAb 4B2-based immunosensor. The responses were reference-subtracted, solvent-corrected, and blank-deducted. Data points are mean ± standard deviation (n = 6, LOD = 0.096 ng mL^{-1}, S/N = 3).

SPR response is the measurement of the refractive index change at the sensor surface, but it does not distinguish between analyte binding to the surface and the differences in the bulk refractive index from the sample solution and the running buffer. Therefore, binding stability levels for concentration analysis should be confidently measured shortly after the end of sample injection, i.e., when the running buffer is flowing over the chip surface and the dissociation phase starts (*Biacore Concentration Analysis Handbook BR-1005-12 Edition AB. In.*, n.d.). Accordingly, the dissociation rate of the immunocomplex is crucial for the binding stability and it can be considered as the key parameter for antibody selection. Via this study of non-competitive direct SPR assay, the differences in binding kinetics of the two anti-triazophos mAbs were observed for the first time, though both mAbs showed similar apparent reactivity in traditional competitive immunoassays such as iELISAs. Previous works on measuring kinetics of hapten-specific antibodies were usually through their interaction to the hapten-protein conjugates immobilized on the sensor chips (Sanders et al., 2016; Hu et al., 2015). However, the hapten-protein conjugate is polyvalent and of great heterogeneity due to the random coupling chemistry. The kinetics and affinity determined by this format is not as straightforward as that from the direct detection of antibody recognition towards a single molecule of LMW analyte. In addition, the formation of stronger bivalent immunocomplex between the antibody and the polyvalent hapten-protein conjugate sometimes brings difficulty for surface regeneration, which was found in our preceding work (Liu et al., 2016c) and previous literatures (Hirakawa et al., 2015b; Munoz et al., 2011). Above all, using an antibody-immobilized direct SPR assay provides better kinetics information of the antibody's activity to LMW target, which is crucial to the antibody profiling and helps to select hapten-specific antibodies for different immunoassays.

For concentration analysis, high RU values are necessary for the assay sensitivity. The use of good-quality antibodies with high affinity towards the target analytes is pivotal to achieve high SPR signal, which help the assay to reach a low LOD. Herein, the mAb 4B2 with relatively slow dissociation rate was used for direct immunosensing of triazophos. Compared to other kinds of competitive immunoassays for rapid and sensitive detection of trizophos residue (Table 5), the newly established non-competitive SPR immunosensor presented a low LOD close to the antibody-coated competitive immunoassays (Jin et al.,

Table 3
Recovery results of triazophos in spiked samples using the SPR immunosensor.

Sample	Matrix dilution	Spiked[a] (ng mL^{-1}; ng g^{-1})	Detected[b] (ng mL^{-1}; ng g^{-1})	Mean recovery (%)	Coefficient of variation (%)
Surface water	10-fold	10	10.65 ± 1.07	106.5	10.1
		50	46.17 ± 3.68	92.3	8.0
Ground water	10-fold	10	10.83 ± 1.23	108.3	11.4
		50	45.04 ± 4.02	90.1	8.9
Chinese cabbage	10-fold	10	10.92 ± 1.18	109.2	10.8
		50	45.66 ± 3.74	91.3	8.2
Cucumber	10-fold	10	10.73 ± 0.99	107.3	9.2
		50	42.18 ± 4.15	84.4	9.8
Apple	20-fold	20	18.64 ± 2.06	93.2	11.1
		100	88.03 ± 8.41	88.0	9.6

[a] The unit of analyte concentration was ng mL^{-1} for water samples, and ng g^{-1} was for other samples.
[b] Detected concentrations were mean ± standard deviation (n = 3).

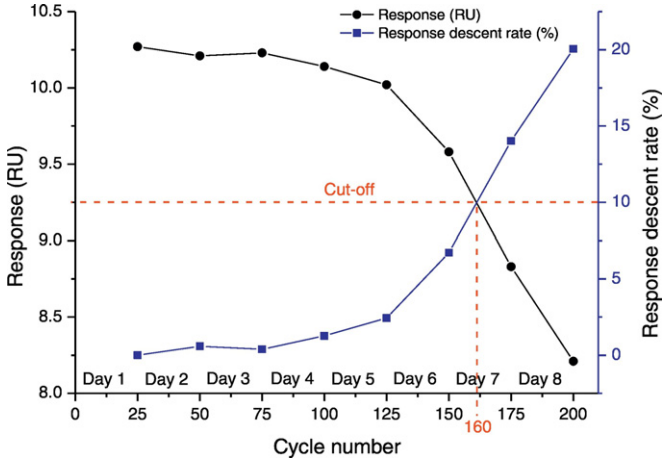

Fig. 5. Stability of the mAb 4B2-based immunochip by repeatedly recording the SPR response of trizophos (32 ng mL^{-1}) at interval of 25 cycles per day (data are the mean values of 3 replicates). The response descent rate was calculated from the record after 25 regeneration cycles on Day 1. Given the cut-off of the descent rate as 10%, the chip could keep effective for 160 cycles within 7 days.

Table 4
Analysis of triazophos residues in unknown real-life samples by the SPR assay and GC–MS.[a]

Sample	No.	Detected by SPR (ng g^{-1})[b]	Detected by GC–MS (ng g^{-1})
Chinese cabbage	1	ND[c]	ND
	2	14.66 ± 1.87	13.97 ± 1.22
	3	ND	ND
	4	21.2 ± 2.51	20.8 ± 1.94
	5	ND	ND
	6	ND	ND
Cucumber	1	ND	ND
	2	ND	ND
	3	32.17 ± 2.76	33.4 ± 2.58
	4	ND	ND
	5	11.83 ± 2.04	11.32 ± 1.55
	6	42.4 ± 5.18	40.95 ± 4.27
Apple	1	ND	ND
	2	ND	ND
	3	64.72 ± 6.86	67.58 ± 6.34
	4	ND	ND
	5	26.35 ± 3.17	27.54 ± 2.68
	6	ND	ND

[a] The limits of detection/quantification (LODs/LOQs) by GC–MS were 2.18/7.26, 2.51/8.37, and 3.77/12.45 ng g^{-1} for triazophos in Chinese cabbage, cucumber and apple, respectively.
[b] Detected concentrations were mean ± standard deviation (n = 3).
[c] ND (not detectable), less than the corresponding LOD.

Table 5
Comparison with other rapid immunoassays for detection of triazophos in buffer.

Assay format[a]	LOD (ng mL^{-1})	Linear range[b] (ng mL^{-1})	Assay time	Ref.
Antibody-coated ELISA	0.07 (IC_{10})	0.12–3.58	75–80 min per run	(Jin et al., 2008)
Antibody-coated enhanced CLEIA	0.063 (S/N = 3)	0.12–6.45	60–65 min per run	(Jin et al., 2012)
An immunomagnetic bead-based assay	0.1 (IC_{10})	0.19–6.37	40–45 min per run	(Du et al., 2015)
A one-step immunogold strip test	4 (By naked eye)	Not determined	10 min per strip	(Du et al., 2015)
Fluorescence polarization immunoassay	0.29 (IC_{10})	1.25–10.48	10 min per run	(Liu et al., 2016a)
Non-competitive piezoelectric immunosensor	40 (S/N = 3)	100–100,000 (Undefined)	40–45 min per cycle	(Huang et al., 2010)
Non-competitive direct SPR immunosensor	0.096 (S/N = 3)	0.98–8.29 (EC_{20}–EC_{80})	7 min per cycle	This work

[a] Only one step of immunoreaction without adding secondary antibody for signal amplification.
[b] Linear range is defined as IC_{20}–IC_{80} or EC_{20}–EC_{80}.

2008; Du et al., 2015; Jin et al., 2012). The linear range, however, was relatively narrow, not as wide as some enzymatic inhibition biosensors developed for organophosphorus pesticides (Ju et al., 2015; Du et al., 2007; Campanella et al., 1996), which might restrict the application of this immunosensor. The drawback was probably related to the limited signal response of binding between the mAb and the LMW analyte (see Fig. 4), since the present one-step direct SPR assay is label-free and without signal enhancement. In fact, for practical application of this method, sample dilution with the running buffer is necessary to remove the matrix influence, so that the triazophos concentration in pretreated samples for injection would not be too high.

Combined with QuEChERS sample pretreatment and appropriate dilution, the newly-developed SPR immunosensor can be used for direct detection of the LMW analyte triazophos in complex food samples, which is usually very challenging. In future, more applications of the biosensor immunoassay for monitoring triazophos residue in other biological samples will be further investigated and extended to other research fields such as occupational epidemiological study and forensic toxicological analysis (Yáñez-Sedeño et al., 2014).

5. Conclusions

To summarize, we proposed a simple, fast, and sensitive diagnostic method for trace triazophos via non-competitive direct SPR immunosensing for the first time. The assay was specific and sensitive with the LOD of 0.096 ng mL^{-1} to triazophos, and the sensor chip could be reused for >160 cycles. Application of real-life sample tests indicated that the novel immunosensor could be used as a convenient and reliable tool of monitoring triazophos residue in the environment and agro-products. This study also paved the way for the development of non-competitive SPR immunosensors for other LMW compounds.

The immunosensor combines the advantages of conventional immunoassays with a high degree of automation, the rapid generation of real-time response, and the sparing use of bio-reagents. Without the competition, pesticide derivatives (haptens) used in the traditional immunoassays as competitors were exempt, thereby avoiding the costly synthesis and the secondary pollution from the experiments. Additionally, SPR kinetic analysis of the interaction between the antibody and the pesticide target offers more binding information, which is beneficial to antibody selection for diverse immunoassay formats.

Note that, the limitations of this study will lead to the further works. Concerning the narrow linear detection range of the SPR assay, other new bio-recognition elements like recombinant antibodies with small sizes could be immobilized to the chip surface and developed for non-competitive immunoassays. Moreover, given that the cost of Bicaore T200 SPR instrument is rather high, the immunosensing method will be exploited to other miniaturized SPR devices in future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.09.157.

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