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Competitive and noncompetitive immunoassays for the detection of benzothiostrobin using magnetic nanoparticles and fluorescein isothiocyanate-labeled peptides

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Abstract

Phage-displayed peptides have been proven to be powerful reagents for competitive and noncompetitive immunoassays. However, they are unconventional reagents, which greatly limit their analytical commercial applications and require additional reagents for detection. In this work, the peptides that specifically bind with anti-benzothiostrobin monoclonal antibody (mAb) or benzothiostrobin-mAb immunocomplex were synthesized and conjugated with fluorescein isothiocyanate (FITC) as substitutes of the phage-displayed peptides to avoid their shortcomings and extend their applications. Competitive and noncompetitive fluorescence immunoassays (FIAs) for benzothiostrobin were developed by mAb coupling with magnetic nanoparticles as concentration elements and peptides conjugated with FITC as tracers. Compared with enzyme-linked immunosorbent assays, the FIAs reduced the number of steps from 6 to 2 and analysis time from more than 5 to 1.2 h. The competitive FIA showed the half-maximal inhibition concentration (IC_{50}) of 16.8 ng mL^{-1} and detection range (IC_{10}–IC_{90}) of 1.0–759.9 ng mL^{-1} , while the concentration of analyte producing 50% saturation of the signal (SC_{50}) and detection range (SC_{10}–SC_{90}) of noncompetitive FIA were 93.4 and 5.9–788.2 ng mL^{-1} , respectively. The average spiked recoveries were 68.33–98.50% and 73.33–96.67% for competitive and noncompetitive FIAs, respectively. The FIAs showed good correlation with high-performance liquid chromatography for the detection of benzothiostrobin in authentic samples.

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Compliance with ethical standards
Conflict of interest The authors declare that they have no conflict of interest.

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Keywords

Pesticide residue; Immunoassay; Peptidomimetic; Immunocomplex; Benzothiostrobin

Introduction

Immunoassay techniques that have well-known advantages of simplicity, fast detection, low cost, and large parallel-processing capacity are widely used for detecting small molecular compounds including pesticides, biological toxins, and antibiotics [1–4]. In general, the immunoassays for small molecular compounds are developed using the reagents of antibody, antigen, and tracer through a competitive format. Phage-displayed peptide libraries are a powerful tool for the isolation of receptor peptides for small molecules [5–7], and ligand peptides for antibodies and enzymes [8–10]. Recently, phage-displayed peptides that specifically bind to antibodies (peptidomimetics) or antigen-antibody complexes (anti-immunocomplex peptides) have been successfully isolated to develop competitive immunoassays or noncompetitive immunoassays for the detection of small molecule compounds [11–14]. However, the peptides are expressed on phage coat protein, which increases their size (880 × 6–7 nm), lower their diffusion rate, and cause a potential biological danger to other biological materials or systems in the laboratory. Additionally, most phage immunoassays need the addition of a second labeled reagent for detection, which increases the number of steps and the total time to complete the assay. All of these shortcomings limit the application of phage-displayed peptides in immunoassays.

Synthetic peptides are relatively cheap materials that can be easily modified to possess a detection or binding element [15]. Previous studies have constructed peptides bound to biotin [16–18], fluorescein isothiocyanate (FITC) [19, 20], and gold nanoparticles [21]. Using these reagents, the assay sensitivities and peptide functionalities are not affected [16, 22], and the detection process can be completed within fewer steps and in less time [15, 18–20]. Therefore, synthetic peptide is a feasible strategy for the use of peptides isolated from phage display libraries in phage-free immunoassays.

In our previous work, both competitive and noncompetitive phage enzyme-linked immunosorbent assays (ELISAs) for benzothiostrobin were developed by using phage-displayed peptides of C3–3 and N6–18 that specifically bind to an anti-benzothiostrobin monoclonal antibody (mAb, $4E_8$) and a benzothiostrobin-mAb immunocomplex, respectively. Benzothiostrobin is a novel strobilurin fungicide, which shows excellent disease control in crops, especially for powdery mildew and downy mildew [23–26]. The half maximal inhibitory concentration (IC_{50}) of the competitive phage ELISA and the concentration of analyte producing 50% saturation of the signal (SC_{50}) of the noncompetitive phage ELISA were 0.94 and 2.27 ng mL^{-1}, respectively [11].

In this study, we tested the hypothesis that these already identified phage-displayed peptides could be conjugated to a reporting element and used for the development of phage-free immunoassays. The conjugations of peptides to FITC were designed and synthesized based on the peptide sequences in the previous study [11]. Both competitive and noncompetitive fluorescence immunoassays (FIAs) for benzothiostrobin were developed by using mAb-conjugated magnetic nanoparticles (MNPs) as concentration elements and FITC-labeled synthetic peptides as tracers. After systematical optimization, the sensitivity, selectivity, precision, and accuracy of the FIAs were evaluated by standard curve, cross-reactivity (CR), and spiked recovery analyses. Additionally, the FIAs were applied to detect residual benzothiostrobin in authentic samples, and the results were validated by high-performance liquid chromatography (HPLC).

Experimental

Reagents and instruments

Benzothiostrobin (97.0%) was a gift from the Central China Normal University (Wuhan, China). The benzothiostrobin analogues pyraclostrobin (99.0%), azoxystrobin (98.5%), kresoxim-methyl (98.0%), picoxystrobin (99.0%), and chloropiperidine ester (96.0%) were purchased from Dr. Ehrenstorfer GmbH (Germany). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Black polystyrene 96-well microtiter plates with a nonbinding surface treatment were purchased from Corning Costar Corporation (NY, USA). Anti-benzothiostrobin mAb 4E_8 [27] and MNPs [28] were prepared previously and stored in the laboratory. The phage-displayed peptides (C3–3, CSGLAEFMSC and N6–18, CPDIWPTAWC) were isolated from a cyclic 8-amino-acid random peptide library previously [11]. FITC was labeled on the N-terminus of the cyclic peptides through aminohexanoic acid (Ahx) as the spacers, which were synthesized and purified by Apeptide Co., Ltd. (Shanghai, China). The sequences of unlabeled and labeled peptides were shown in the Electronic Supplementary Material (ESM) Table S1. All other chemical reagents were of analytical grade.

The fluorescence intensity was detected by a Molecular Devices SpectraMax M5 (San Jose, CA, USA). The zeta potential of MNPs was detected by Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK). Benzothiostrobin was detected using an Agilent 1260 HPLC (Santa Clara, CA, USA). MNPs were separated by an Alrun MS-12 magnetic separator (Shanghai, China).

Preparation and verification of mAb-conjugated MNPs

The mAb was conjugated to the MNPs according to the method previously described [28]. Briefly, 10 mg of MNPs were thoroughly dispersed in 5 mL of 0.05 M, pH 7.4 sodium borate buffer (BB) by ultrasonication for 1 h. Subsequently, 100 mg sodium borohydride ($NaBH_4$) and 1.25 mL of glutar-aldehyde (25%) were added and the mixture was shaken for 1 h. The MNPs were separated by a magnetic separator and washed three times with BB. Then, the MNPs were again dispersed in 5 mL BB and 0.8 mg of anti-benzothiostrobin mAb was added and shaken for 6 h. The unreacted sites were blocked by the addition of 5 mL of 1% BSA and rotated with gentle rocking for 6 h. After separation and washing, the mAb-conjugated MNPs were dispersed in 5 mL BB and stored at 4 °C.

The mAb-conjugated MNPs were identified by goat antimouse IgG-horse radish peroxidase (HRP) and surface chemistry [29]. For the goat anti-mouse IgG-HRP determination, 400 μL BB, 200 μL goat anti-mouse IgG-HRP (1:20000 in BB) and 200 μL mAb-MNPs were added to 2-mL tubes for incubation of 1 h at 37 °C. After separation and washing three times with BB, 500 μL substrate solution (25 mL of 0.1 M citric acid and dibasic sodium phosphate buffer (pH 5.5), 0.1 mL of 3 mM H_2O_2, 0.4 mL of 25 mM tetramethylbenzidine in dimethyl sulfoxide) was added to detect the bound enzyme (HRP). The MNPs that were blocked by BSA were set as a control. The average optical density (OD) values at 650 nm were detected by a SpectraMax M5.

Immunoassay protocols

For the competitive FIA, benzothiostrobin competes with the tracer (FITC-Ahx-CSGLAEFMSC, FITC-C3–3) to bind with the mAb-MNPs, the fluorescence signal is decreased with the increase of the concentration of benzothiostrobin (Fig. 1a). For the noncompetitive FIA, the tracer (FITC-Ahx-CPDIWPTAWC, FITC-N6–18) specifically binds to the benzothiostrobin-mAb-MNP immunocomplex, the fluorescence signal is positively correlated with the concentration of benzothiostrobin (Fig. 1b). The procedural steps for the competitive and noncompetitive FIAs used the same volumes and conditions. Four hundred microliters of standard or sample solution, 200 μL of tracers (20 μg mL^{-1} of FITC-C3–3 or 40 μg mL^{-1} of FITC-N6–18), and 200 μL of mAb-MNPs were added to a 2-mL tube and shaken for 1 h. The MNPs were separated by an external magnet and washed three times with BB. Then, 100 μL elution solution (2% sodium dodecyl sulfate, SDS) was added and incubated for 10 min with slow rocking. Again, the MNPs were separated by an external magnet and 100 μL of the supernatant was transferred into a 96-well plate and the fluorescence intensity was measured using a SpectraMax M5 at excitation and emission wavelengths (λ_{ex}, λ_{em}) of 492 nm and 525 nm. The binding rate (B/B_0) between the tracers and antibody (or immunocomplex) was calculated as a percentage according to the formula: $B/B_0 = (F - F_{min})/(F_{max} - F_{min}) \times 100\%$ (F represents measured fluorescence intensity, F_{max} represents fluorescence intensity at the maximum tracers binding with antibody or immunocomplex, F_{min} represents fluorescence intensity at the minimum tracers binding with antibody or immunocomplex).

Optimization of FIAs

Six different elution solutions including 0.2 M glycine hydrochloride (Gly-HCl, pH = 2.2), 0.15 M sodium chloride (NaCl) (pH = 11), 5 M magnesium chloride ($MgCl_2$), 50% glycol, 8 M urea, and 2% SDS and elution time (0, 5, 10 15, 20, 25, and 30 min) were evaluated by measuring their influence on fluorescence intensity and stability. A series of volumes of mAb-conjugated MNPs (25, 50, 100, and 200 μL) and concentrations of tracers (5, 10, 20, 40, 80, and 160 μg mL^{-1}) were optimized in the immunoassays. The assessment of competitive immunoassay was based on F_{max}/IC_{50}, while the noncompetitive immunoassay was based on the ratio of fluorescence intensity in the presence (200 ng mL^{-1}) and absence of benzothiostrobin, with higher values being the most desirable. The immunoassays were optimized by testing buffers with various pH values (4.4, 5.4, 6.4, 7.4, 8.4, and 9.4), concentrations of Na^+ (0.1, 0.2, 0.3, 0.4, and 0.5 M), and content of methanol (2.5%, 5%, 10%, and 20%). The parameters resulting in the highest F_{max}/IC_{50} (or F_{max}/SC_{50}) were the most desirable.

CRs

The analogues of benzothiostrobin, including pyraclostrobin, azoxystrobin, kresoxim-methyl, picoxystrobin, and chloropiperidine ester, were tested for CR in the immunoassays. The CRs were calculated based on the IC_{50} (or SC_{50}) values according to the following formula: CR (%) = [IC_{50} (or SC_{50}) (benzothiostrobin)/IC_{50} (or SC_{50}) (analogue)] × 100.

Analysis of spiked samples

Cucumber, rice, and corn were purchased from the supermarket in Nanjing, China. Paddy water and soil were obtained from the farm in Nanjing, China. Paddy water was filtered through a 0.22-μm filter and spiked with benzothiostrobin. The solid samples (soil, cucumber, rice, and corn) (10 g) were homogenized and spiked with benzothiostrobin. The final concentrations of the spiked samples were 0.1, 0.3, and 1.0 mg L^{-1} for paddy water and 0.3, 1.0, and 5.0 mg Kg^{-1} for solid samples. Paddy water was analyzed directly after mixing with isometric 2× optimal buffer. The solid samples (10 g) were extracted using 20 mL of BB containing 25% methanol, vortexed for 3 min, and sonicated for 15 min. After centrifugation at 4000 rpm for 5 min, the supernatant was transferred and adjusted to 25 mL using BB. The solutions were diluted appropriately and detected by the immunoassays.

HPLC analysis and validation

Authentic samples of cucumber were collected from the farms in Nanjing, China, where benzothiostrobin had been sprayed. The amounts of benzothiostrobin in these authentic samples were simultaneously analyzed by the immunoassays and HPLC. For the immunoassays, the extraction and analysis of these samples were the same as those of the spiked samples.

For HPLC, 10 g homogenized samples were extracted by 50 mL acetonitrile with vigorous shaking for 1 h. The mixture was moved to a 100-mL cylinder with stopper, and the organic phase was separated by the addition of 5 g NaCl. Half of the organic phase was collected and dried at 50 °C under nitrogen. The concentrate was dissolved in 6 mL n-hexane/acetone (95/5, *v/v*) and loaded on a Florisil SPE column which was activated by 6 mL n-hexane. Next, the column was eluted by 10 mL n-hexane/acetone (80/20, *v/v*), and the eluate was collected and evaporated to dryness. Finally, the extract was dissolved in 2 mL acetonitrile for the analysis by HPLC (Agilent 1260) with an SB-C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was acetonitrile/water (65/35, *v/v*) and the flow rate was 1.0 mL min^{-1} at 30 °C. The detection wavelength was 230 nm and the injection volume was 20 μL.

Results and discussion

Identification of mAb-conjugated MNPs

In the goat anti-mouse IgG-HRP determination, the substrate solution color of mAb-MNPs was dark blue, which was different with the blocked MNPs (brown) (ESM Fig. S1a). The average OD values of blocked MNPs and mAb-MNPs were 1.63 ± 0.09 and 2.51 ± 0.13, respectively (ESM Fig. S1b). Besides, the average zeta potential of MNPs changed from − 42.67 ± 2.26 mV to − 33.50 ± 1.69 mV after coupling with the mAb ($n = 3$). These results indicated that the mAb was conjugated to MNPs.

Elution solution and elution time

As shown in Fig. 2a, the absorption spectrum of MNPs is overlapped with the fluorescence spectra (excitation and emission spectrum) of tracers. The fluorescence intensities of tracers in BB without MNPs were markedly higher than those with MNPs (Fig. 2b), because a part of excitation and emission spectra were absorbed by MNPs due to the inner filter effect. Therefore, the fluorescence intensity was measured after the elution in the process of the immunoassays. Six different elution solutions (pH 2.2, 0.2 M Gly-HCl; pH 11, 0.15 M NaCl; 5 M $MgCl_2$; 50% glycol; 8 M urea; 2% SDS) were used to elute tracers by incubation for 20 min, the fluorescence intensity was enhanced greatly when 8 M urea or 2% SDS was applied for the elution (Fig. 2c). Due to the higher fluorescence intensity, 2% SDS was selected as the optimal elution solution. The effect on FITC fluorescence intensity by six elution solutions was shown in Fig. 2d, the fluorescence intensity was reduced by Gly-HCl, $MgCl_2$, and glycol. Besides, urea and SDS have proved to be more capable of elution [30]. Therefore, the difference between six elution solutions was mainly caused by elution ability and the effect of elution solution on fluorescence intensity. Figure 2e shows the fluorescence intensity at different elution time points, where the fluorescence intensity reached the maximum and stabilizes after incubation for 10 min. Therefore, the optimal elution time was set at 10 min.

Optimization of concentrations of tracers and mAb-conjugated MNPs

Appropriate concentrations of antibody and tracer are very important for the sensitivity of the immunoassay. In our experiment, different methods were chosen to obtain the optimal concentration of antibody and tracer. For competitive FIA, the fluorescence intensity with 20 μg mL^{-1} tracer FITC-C3–3 and different volumes of mAb-conjugated MNPs were shown in ESM Fig. S2a, fluorescence intensity increased with the increase of mAb-conjugated MNPs, so 200 μL was selected as the optimal dosage. ESM Fig. S2b shows the IC_{50} and F_{max}/IC_{50} under the concentrations oftracer from 5 to 160 μg mL^{-1}, where 20 μg mL^{-1} was selected based on the lower IC_{50} and higher F_{max}/IC_{50}. For noncompetitive FIA, the maximal signal difference in the presence and absence of benzothiostrobin (200 ng mL^{-1}) was observed at mAb-conjugated MNPs volume of 200 μL and tracer (FITC-N6–18) concentration of 40 μg mL^{-1} (ESM Fig. S3).

Optimization of assay buffer

In order to improve the performance of immunoassays, the parameters of pH, ionic strength, and methanol content were optimized. As shown in ESM Fig. S4, pH 7.4, 0.1M Na^+, and 2.5% methanol were selected as optimal conditions for competitive FIA because it showed the highest F_{max}/IC_{50}, while the optimal conditions for noncompetitive FIA were pH 7.4, 0.3 M Na^+, and 2.5% methanol (ESM Fig. S5).

Sensitivity

The standard cures of competitive and noncompetitive immunoassays for benzothiostrobin were established by plotting the B/B_0 versus the logarithm concentration of benzothiostrobin under the optimal conditions (Fig. 3). The IC_{50}, limit of detection (LOD, IC_{10}) and detection range (IC_{10}–IC_{90}) of competitive FIA were 16.8, 1.0, and 1.0–759.9 ng mL^{-1}, while SC_{50}, LOD (SC_{10}), and detection range (SC_{10}–SC_{90}) of the noncompetitive FIA were 93.4, 5.9, and 5.9–788.2 ng mL^{-1}, respectively. Compared to our previous immunoassays using the same mAb, the conventional ELISA (the coating antigen was prepared by hapten) [27] and phage ELISAs [11], this assay was approximately 5-fold less sensitive according to LOD values (Table 1). Although the sensitivities of the FIAs were lower than the reported methods, their sensitivities were acceptable and their detection ranges were broader. In addition, the FIAs had obvious advantages in the reduced number of steps (from 6 steps down to 2) and reduction in overall time (from more than 5 to 1.2 h) to complete the analysis.

The conventional ELISA and phage ELISA generally have higher sensitivity because a large amount of tracer (typically an antibody coupled to HRP) can be bound to the extended surface of the antibody in conventional ELISA and the phage in phage ELISA to enhance the detection signal [13]. This enhanced signal is lost when the peptide is transferred from the phage to FITC. Therefore, the sensitivities of the FIAs were slightly lower than ELISAs. These results indicated that the strategy of synthetic peptide tracer did not affect the activity of the peptide, and could avoid the shortcomings associated with phage's large size and biological nature. Besides, the peptide-FITC could be directly used as tracers to avoid the need of additional reagents for detection (antibody coupled to HRP in ELISAs), which made the FIAs simpler and faster than conventional ELISA and phage ELISA. Therefore, the synthetic peptide tracers were efficient substitutes of the phage-displayed peptides to avoid their shortcomings and extend their use in phage-free immunoassays with simple and rapid detection procedure.

Selectivity

The CRs for the analogues structurally related to benzothiostrobin were calculated and shown in Table 2. Both the competitive and noncompetitive immunoassays demonstrated no CR with the analogues of benzothiostrobin (the CRs were less than 0.1%), which indicated that the immunoassays had high selectivity for benzothiostrobin. Previously, both the indirect competitive and the phage-based competitive ELISA exhibited slight CR (0.34%) with pyraclostrobin [11, 27].

Analysis of spiked sample

Matrix interferences of samples usually influence the accuracy of immunoassays. Dilution of the sample matrices with buffer is a simple and effective method to eliminate the influence. The matrix interference of paddy water was investigated by performing no dilution, 2-fold dilution, and 4-fold dilution after mixing with isometric 2× optimal buffer (the total dilution was 4-, 8-, and 16-fold, after mixing with tracer and mAb-MNPs in the immunoassay procedures). The matrix interferences of the solid samples (including soil, cucumber, rice, and corn) were investigated by performing a 2-, 4-, and 8-fold dilution with BB (the total dilution was 10-, 20-, and 40-fold, after extraction and mixing with tracer and mAb-MNPs in the immunoassay procedures). The diluted matrices were used to establish standard curves of benzothiostrobin by the FIAs. A 4-fold dilution of paddy water, a 20-fold dilution of soil and corn matrices, and a 40-fold dilution of cucumber and rice matrices were selected for competitive and noncompetitive FIAs, because the standard curves produced from the diluted matrices were similar to those from BB (ESM Figs. S6 and S7).

Based on the dilutions, the spiked samples were tested by the FIAs. The average recoveries and RSDs were calculated and summarized in Table 3. The average recoveries of competitive FIA ranged from 68.33–98.50% with RSDs of 2.12–13.91%, while the noncompetitive FIA ranged from 73.33–96.67% with RSDs of 2.83–14.14%. These results indicated that the accuracy and precision of these immunoassays for detection using this extraction procedure was suitable for the quantitative analysis of benzothiostrobin according to the guideline on pesticide residue trials of China (NY/T 788–2004).

Validation with HPLC

Authentic cucumber samples were analyzed simultaneously by the FIAs and HPLC, the amounts of benzothiostrobin detected by the FIAs (competitive, 210–2394 ng g^{-1}; noncompetitive, 248–2411 ng g^{-1}) were similar with these detected by HPLC (226–2477 ng g^{-1}) (ESM Table S2). The P values generated by a Student's t test of competitive and noncompetitive immunoassays compared to HPLC were 0.882 and 0.938 (greater than 0.05), which implied the data between the FIAs and HPLC were not significantly different. In addition, Fig. 4 showed the correlations between the FIAs and HPLC for the authentic cucumber samples, wherein the slope and R^2 values were close to 1. These results indicated that the presented immunoassays were reliable and accurate for the determination of benzothiostrobin in authentic cucumber samples exposed to benzothiostrobin.

Conclusions

In this study, the synthetic peptide tracers were used as the substitutes of the phage-displayed peptides that isolated from a phage display peptide library to develop competitive and noncompetitive FIAs by using MNPs as the reaction platform. This is a significant contribution to the field of immunoassays for small molecular compounds. Firstly, the inner filter effect caused by MNPs as the reaction platform was explained, and the elution solutions and elution time were optimized. Secondly, synthetic peptide tracers avoided the shortcomings of the phage particles. Thirdly, the immunoassays had obvious advantages in the reduced number of steps and reduction in overall time to complete the analysis. To the best our knowledge, the competitive and noncompetitive FIAs were developed by using synthetic peptides and MNPs for the first time. With the growing use of phage display peptide libraries for the isolation of peptidomimetics and anti-immunocomplex peptides, we believe that synthetic peptide tracers and FIAs based on MNPs will be the useful addition to the analytical toolbox.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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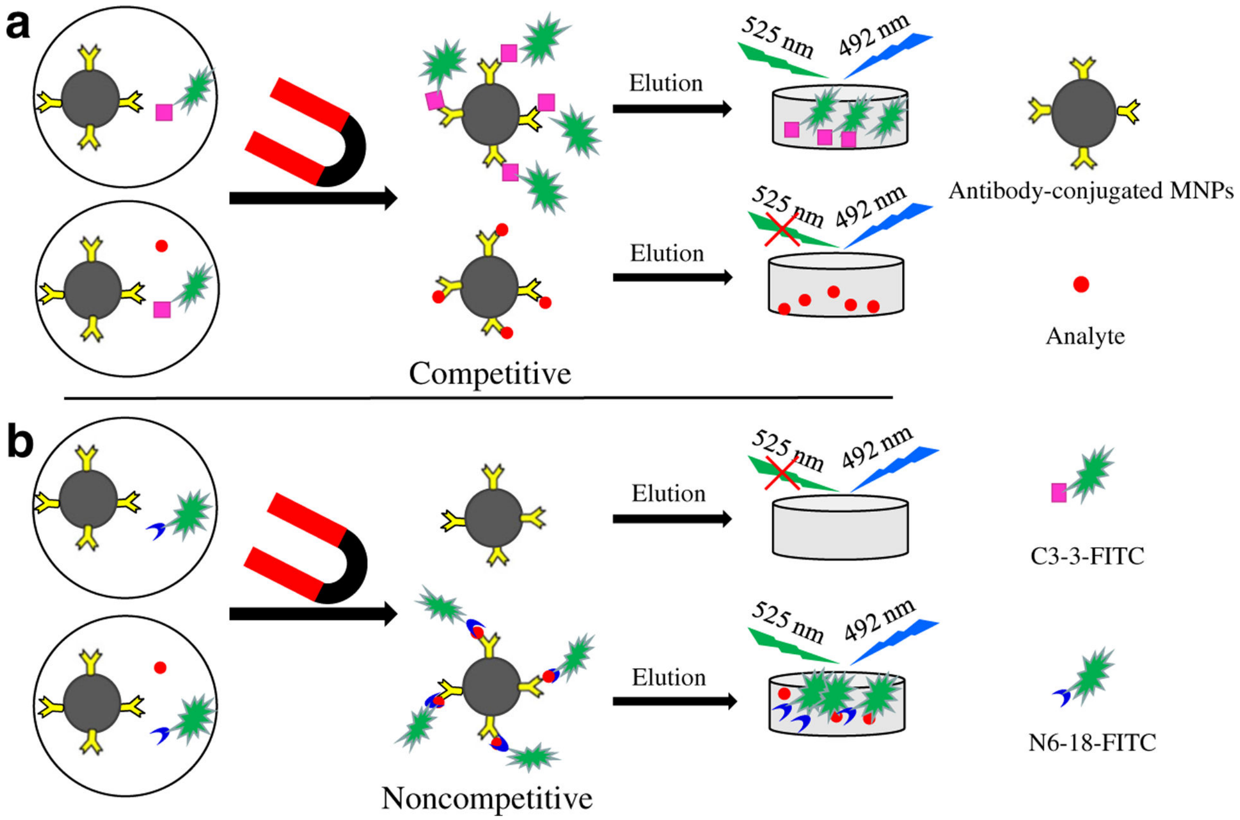


Fig. 1.

Schematic representations of competitive FIA (**a**) and noncompetitive FIA (**b**) on the basis of FITC-labeled peptides C3–3 (CSGLAEFMSC) and N6–18 (CPDIWPTAWC)

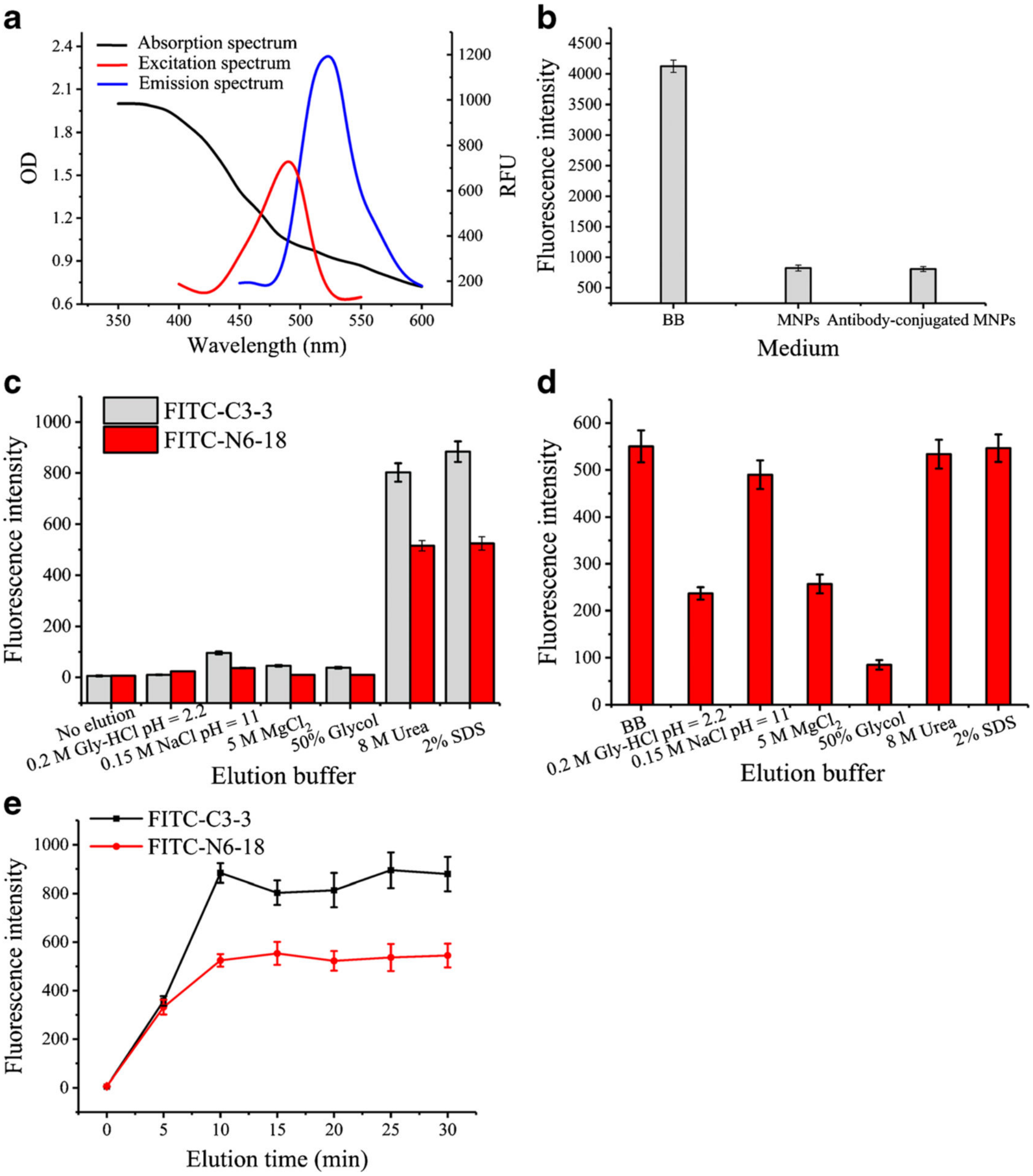


Fig. 2.

The absorption spectrum of MNPs (black curve), and excitation and emission spectra of tracer FITC-C3–3 (red and blue curves) (**a**); the fluorescence intensity of tracer FITC-C3–3 in BB, MNPs, and mAb-conjugated MNPs (**b**); the fluorescence intensity after eluted by 0.2 M Gly-HCl (pH = 2.2), 0.15 M NaCl (pH = 11), 5 M MgCl$_2$, 50% Glycol, 8 M urea, and 2% SDS (**c**); the fluorescence intensity of tracer FITC-C3–3 at same concentration in different elution buffer (**d**); the fluorescence intensity at different elution times with 2% SDS (**e**)

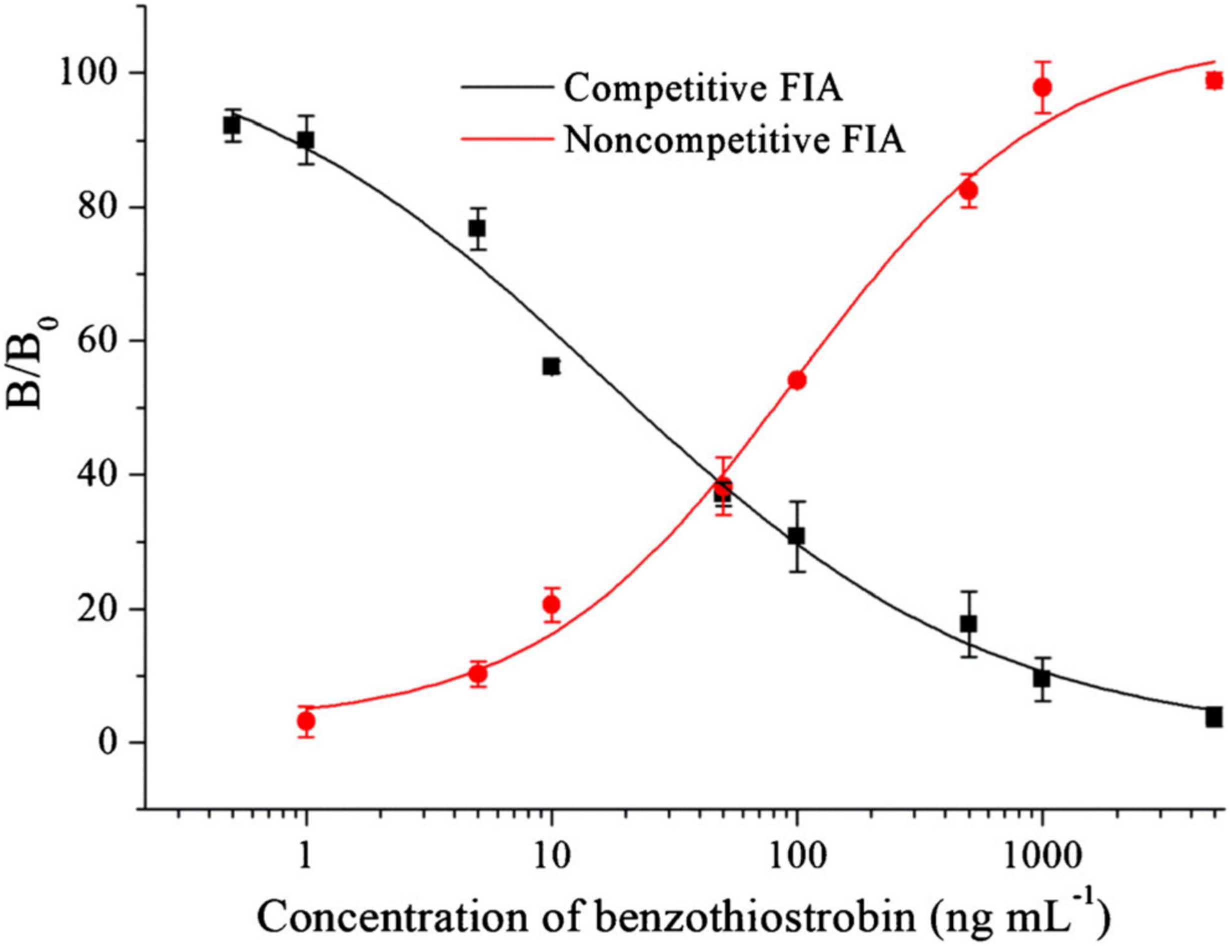


Fig. 3.

Standard curve of benzothiostrobin by the FIAs. Each point represents the average of three repetitions

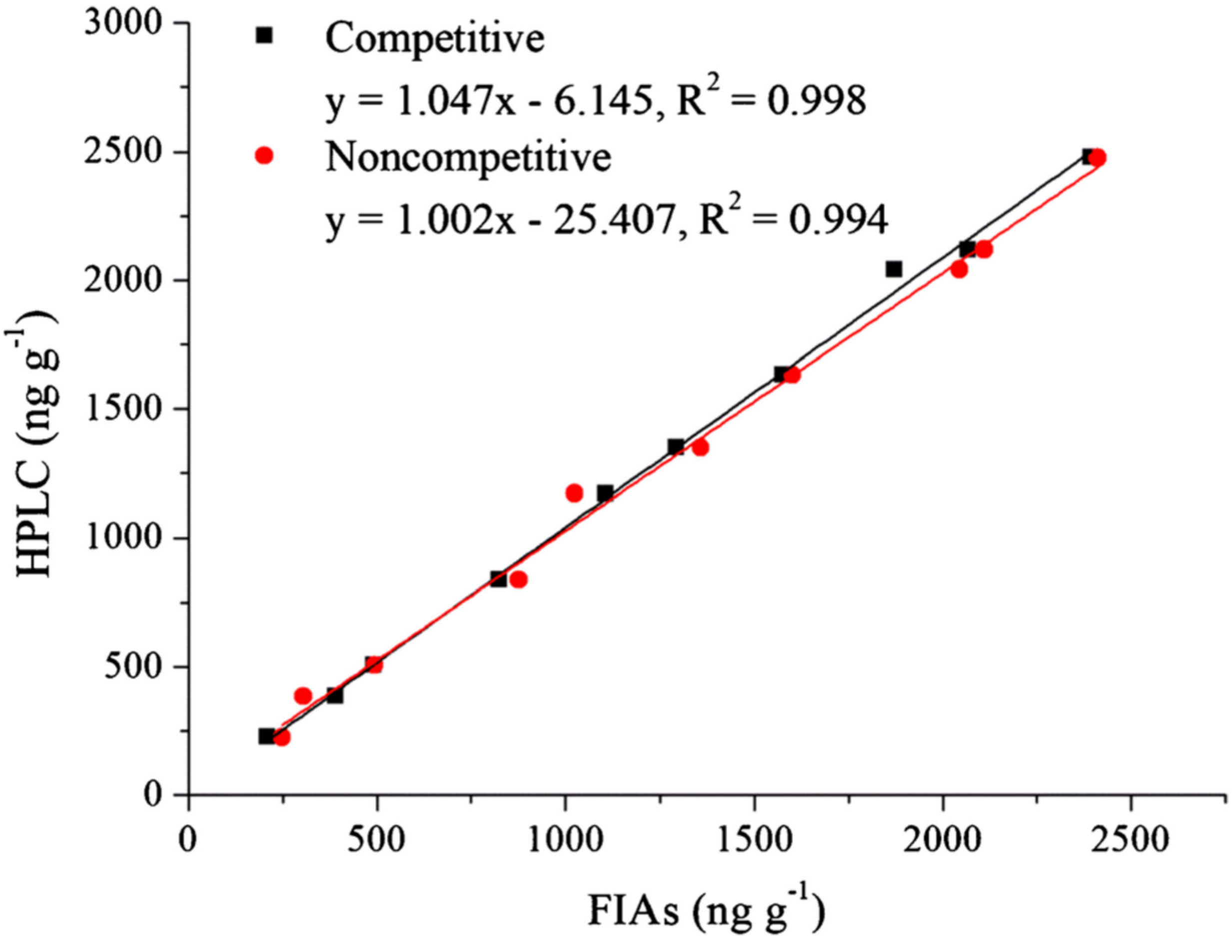


Fig. 4.

Correlations between the FIAs and HPLC for the authentic cucumber samples

Table 1

Comparison of previous and present immunoassays for benzothiostrobin

Methods		IC_{50} or SC_{50} (ng mL^{-1})	LOD (ng mL^{-1})	Detection range (ng mL^{-1})	Time (h)	Step	Reference
Conventional ELISA		7.55	0.43	0.43–54	5.08[†]	6	22
Phage ELISAs	Competitive	0.94	0.22	0.22–3.94	5.75		6
	Noncompetitive	2.27	1.11	1.11–4.62	5.75		
FIAs	Competitive	16.8	1.0	1.0–759.9	1.2	2	This work
	Noncompetitive	93.4	5.9	5.9–788.2	1.2		

[†] The incubation for overnight at 4 °C was replaced by incubation for 2 h at 37 °C to calculate the analysis time

Table 2

Cross-reactivity of analogues structurally related to benzothiostrobin determined by the FIAs

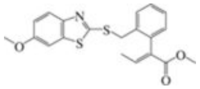
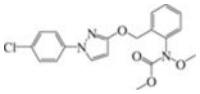
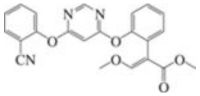
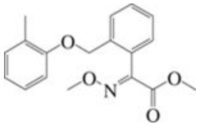
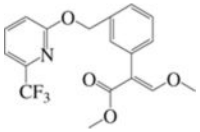
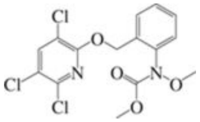
Compound	Chemical structure	Competitive		Noncompetitive	
		IC_{50} (ng mL^{-1})	**CR (%)**	**SC_{50} (ng mL^{-1})**	**CR (%)**
Benzothiostrobin		16.8	100	93.4	100
Pyraclostrobin		>20000	<0.1	>100000	<0.1
Azoxystrobin		>20000	<0.1	>100000	<0.1
Kresoxim-methyl		>20000	<0.1	>100000	<0.1
Picoxystrobin		>20000	<0.1	>100000	<0.1
Chloropiperidine ester		>20000	<0.1	>100000	<0.1

Table 3

Average recoveries of samples spiked with benzothiostrobin by FIAs (*n* = 3)

Samples	Spiked (mg L^{-1} or mg Kg^{-1})	Competitive		Noncompetitive	
		Average recovery (%)	RSD (%)	Average recovery (%)	RSD (%)
Paddy water	0.1	90.2	13.9	88.3	7.1
	0.3	98.5	13.4	81.0	11.3
	1.0	97.3	8.1	83.8	3.4
Soil	0.3	76.8	9.7	96.7	9.4
	1.0	78.6	6.4	77.5	5.0
	5.0	91.7	3.5	90.7	8.3
Cucumber	0.3	96.5	10.6	73.3	14.1
	1.0	79.7	5.2	81.0	2.8
	5.0	85.8	6.2	86.6	6.5
Rice	0.3	68.3	7.1	93.3	4.7
	1.0	91.0	4.2	82.5	12.0
	5.0	92.8	3.7	85.9	6.7
Corn	0.3	80.2	9.2	83.4	9.4
	1.0	78.1	5.7	83.5	7.8
	5.0	77.7	2.1	84.8	3.8