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Highly Specific Monoclonal Antibody and Sensitive Quantum Dot Beads-Based Fluorescence Immunochromatographic Test Strip for Tebuconazole Assay in Agricultural Products

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Abstract

A monoclonal antibody (mAb) was raised against tebuconazole (TEB) using a hapten where the *p*-chloro substituent of the TEB molecule was replaced with a long-chain carboxylic acid. The resulting mAb showed high sensitivity and specificity against TEB characterized by ELISA with a half-maximal inhibitory concentration (IC_{50}) of 0.19 ng mL^{-1} and with cross-reactivity (CR) values below 0.01% to several analogues of triazole fungicides. On the basis of the mAb produced, a quantum dot beads-based fluorescence immunochromatographic test strip assay (QBs–FITSA) was developed for rapid and sensitive detection of TEB in agricultural product samples. The QBs–FITSA exhibited a linear detection range from 0.02 to 1.25 ng mL^{-1} with a limit of detection (LOD) of 0.02 ng mL^{-1}. Furthermore, using produced mAb, multiple high-throughput rapid immunoassay formats could be achieved as a convenient monitoring tool for evaluation of human and environmental exposure to TEB.

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ASSOCIATED CONTENT

Supporting Information

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ELISA procedure, positive ion electrospray mass spectra, and ^1H NMR spectra of hapten; UV–vis spectra of hapten and hapten–protein conjugate; MALDI-TOF/TOF spectra of hapten–protein; cross-reactivity study of QBs–FITSA; photographs of tebuconazole detection in 9 real samples; optimization of the concentration of hapten-OVA and the volume of QBs–mAb conjugate; comparison of different methods for the detection of TEB (PDF)

The authors declare no competing financial interest.

Keywords

immunochromatographic test strip; monoclonal antibody; tebuconazole; fluorescence; quantum dot beads

1. INTRODUCTION

Tebuconazole (TEB) [(RS)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl) pentan-3-ol] is a broad spectrum and highly active triazole systemic fungicide developed by Bayer Corp.[1] This compound inhibits fungal cytochrome P450 (CYP) 51 (lanosterol-14α-demethylase). Among other things this interferes with cell membrane synthesis, leading to the inhibition of fungal growth.[2,3] Numerous studies have shown that TEB is moderately toxic for many organisms and is possibly carcinogenic in humans.[4,5] TEB exerts hepatotoxicity in rodent studies and has potential effects on the endocrine system.[6] There is increasing concern over personnel exposure to TEB during manufacture, diet, and drinking water. The European Commission and The Ministry of Health of the People's Republic of China have regulated the maximum residue limits (MRLs) of TEB at 0.05 mg kg^{-1} in some crops and vegetables (Nos. EU 750/2010 and GB-2763–2016).[7,8] Thus, it is critical to develop sensitive, rapid, and efficient analytical methods for both environmental monitoring and food safety.

Immunoassay has emerged as a versatile complementary analytical tool to conventional instrumental analyses for on-site testing and high-throughput screening.[9,10] Conventional instrumental analyses, such as gas chromatography (GC), liquid chromatography (LC), or GC coupled to mass spectrometry (GC-MS),[11,12] show high accuracy and adequate detection limit for TEB residue analysis, but these methods are restricted by high-cost complex equipment requirement and time-consuming laborious sample pretreatment as well as limited highly qualified personnel. Thus, immunoassay is increasingly being recognized as an effective way to make up these deficiencies in terms of simplicity, portability, and cost effectiveness.[13–16]

A high-quality antibody is the key point for developing high-performance immunoassays, and generation of highly functional antibodies to small molecular weight compounds is highly dependent on the proper design of haptens.[17,18] In a previous report Danks et al. developed a specific polyclonal antibody against TEB by using TEB derivatives as haptens.[19] They developed an enzyme-linked immunosorbent assay (ELISA) for TEB with a linear detection range of 0.02–20 μg mL^{-1} and showed negligible or no cross-reactivity to seven other triazole compounds. However, surprisingly, we find there are only a few studies on the immunoassay for TEB,[19,20] not only the limited antibody types but also the immunoassay methods development. On this basis, in this work a high-affinity monoclonal antibody (mAb) was generated against haptens by derivatizing at the *p*-chloro position of the TEB molecule with a $-C_5H_{10}COOH$ spacer arm (Figure 1). A resulting mAb, E3H11, was used to develop a sensitive quantum dot beads-based fluorescence immunochromatographic test strip assay (QBs–FITSA) for the determination of TEB.

The developed QBs–FITSA shows superior performance in terms of both sensitivity and speed of analysis compared to previously reported assays for TEB quantification.[19] These good characteristics can be attributed in part to the outstanding fluorescence properties of quantum dot beads (QBs) and the fast capillary action of the immunochromatographic test strip format as well as the high affinity of mAb. QBs not only have the unique properties of quantum dots (QDs), such as broad adsorption, robust photostability, and symmetric photoluminescence spectra, but also show 1000 times brighter luminescence than the corresponding QDs, which are considered by many as ideal signal labels for high-sensitivity measurement.[21,22] Ren et al. developed a luminescent QBs-based immunochromatographic assay for aflatoxin B_1 with 2 orders of magnitude better sensitivity than those of gold nanoparticle-based immunochromatographic assay and was even comparable with or better than the conventional ELISA method.[22] The immunochromatographic test strip is a powerful analytical format enabling rapid and low sample consumption on-site detection.[23] In addition, the mAb reported here exhibits high affinity and specificity against the target TEB. Thus, by taking all of the advantages mentioned above, the developed QBs–FITSA shows high sensitivity and selectivity for TEB detection and is clarified to be suitable as a rapid and convenient monitoring tool for evaluation of human and environmental exposure to TEB.

2. MATERIALS AND METHODS

2.1. Reagents.

Tebuconazole (TEB), Ovalbumin (OVA), bovine serum albumin (BSA), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbo-diimide hydrochloride (EDC·HCl), dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), tri-*n*-butylamine, isobutyl-chlorocarbonate, complete and incomplete Freund's adjuvant, poly-ethylene glycol (PEG) 2000, hypoxanthine-aminopterin-thymidine (HAT) and hypoxanthine-thymidine (HT) medium, peroxidase-labeled goat antimouse IgGs, 3,3′,5,5′-tetramethylbenzidine (TMB), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Cell culture medium (DMEM) and fetal bovine serum were provided by Thermo Fisher Scientific (Waltham, MA, USA). TEB analogues (penconazole, propiconazole, myclobutanil, paclobutrazol, and hexaconazole) used for cross-reactivity studies were supplied by Aladdin Chemistry Co., Ltd. (Shanghai, China).

Quantum dot beads (QBs) were generated in Prof. Yonghua Xiong's lab (Nanchang University, Nanchang, China).[22] The elements of the immunochromatographic test strip platform comprising sample, absorbent pads, and NC membranes were purchased from Millipore Corp. (Bedford, MA, USA).

2.2. Apparatus.

Cell culture plates and 96-wells polystyrene ELISA plates were purchased from Costar (Corning, NY, USA). ELISA plates were washed with a microplate washer from Thermo Fisher Scientific (Waltham, MA, USA), and ELISA absorbance was obtained by a multifunctional microplate reader from Thermo Fisher Scientific (Waltham, MA, USA). The test strips were prepared using the XYZ 3050 dispensing platform and CM4000 Guillotine

Cutter (BioDot, Irvine, CA). The fluorescence intensity of T line and C line on the QBs–FITSA was detected using a fluorescence immunoassay analyzer FIC-S1 from Helmen Co., Ltd. (Suzhou, China), and the photos of QBs–FITSA were taken by UV-based analyzer ZF-1 from Lichen Co., Ltd. (Shanghai, China).

2.3. Synthesis of Hapten and Antigen.

The designed hapten was synthesized based on the synthetic route illustrated in Figure 1. The hapten was synthesized by substitution of the *p*-chloro substituent of TEB with a $-C_5H_{10}COOH$ spacer arm. Briefly, 5.8 g of TEB (18.8 mmol) was dissolved in 100 mL of dioxane/H_2O (v/v = 5/1), 7.2 g of methyl (*E*)-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl) hex-5-enoate (28.2 mmol), 5.2 g of K_2CO_3 (37.6 mmol), and 1.2 g of $Pd(dtbpf)Cl_2$ (1.9 mmol) were added sequentially and heated at 90 °C for 16 h. Then Pd/C was added to the chilled (on an ice bath) solution of the product in MeOH (vacuum/nitrogen). The resulting mixture was hydrogenated for 16 h and filtered through a short plug of Celite, followed by a rinsing step with MeOH. The combined filtrates were concentrated in vacuum, and the residue was dried overnight. The obtained product was dissolved in EtOH, and 2 M NaOH (3.0 equiv) was added on an ice bath. The mixture was refluxed for 1 h, filtered, and rinsed with water to obtain the hapten product.

The immunogen (Hapten-BSA) was prepared using the active ester method.[24] Briefly, 19.4 mg of hapten, 41.2 mg of DCC, and 11.5 mg of NHS were dissolved in 1 mL of *N*-dimethylformamide (DMF) with stirring overnight at room temperature (RT). After centrifugation, the supernatant was added dropwise into 5 mL of carbonate buffer (100 mM, pH 9.6) containing 82.5 mg of BSA and stirred for 4 h at RT. The product was dialyzed by phosphate buffer (PBS, 10 mM, pH 7.4) and stored at −20 °C.

Coating antigen (Hapten-OVA) was prepared by the mixed-anhydride method.[25] Briefly, 19.4 mg of hapten, 20 μL of trinbutylamine, and 10 μL of isobutylchlorocarbonate were mixed in 1 mL of DMF with stirring 1 h at 4 °C. The mixture solution was added dropwise into 5 mL of carbonate buffer containing 30 mg of OVA and stirred for 2 h at RT. The product was dialyzed by PBS and stored at −20 °C.

2.4. Production of Monoclonal Antibody.

The production of the monoclonal antibody was made following the protocol of a previous report.[24] Briefly, five female BALB/c mice (6 weeks old) were immunized with hapten-BSA. The antigen 100 μg in PBS was emulsified with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously. A 50 μg amount of immunogen emulsified with Freund's incomplete adjuvant (1:1 v/v) were used for the next four sequential booster immunizations over a 3-week interval. The sera of terminal bleeds were used to evaluate the titer and ability of target recognition of antibody by ELISA.

Cell fusion was performed using the classic hybridoma technology according to a previous study.[26] Seven to 10 days after cell fusion, positive hybridoma cell clones were identified by ic-ELISA. Ascites fluids were produced in BALB/c mice primed with paraffin and purified by affinity chromatography on protein G sepharose. The purified antibody products were dialyzed by PBS (10 mM, pH 7.4) and stored at −20 °C.

2.5. Preparation of QBs–mAb.

The QBs–mAb conjugates were prepared by carbodiimide cross-linker chemistry.[22,24] First, 1 μL of QBs (12.5 mg mL^{-1}) was dispersed in 0.5 mL of PBS (10 mM, pH 6.0), and 9 μL of EDC (1 mg mL^{-1}) was added to activate the −COOH groups on the QBs surface with stirring for 30 min at room temperature (RT). Then 8 μL of anti-TEB mAb (0.62 mg mL^{-1}) was added into the mixture for another 30 min stirring at RT. Subsequently, the mixture was stabilized by the addition of 50 μL of BSA (100 mg mL^{-1}) with stirring for 30 min. The solution was centrifuged at 13 500 × *g* for 10 min to remove unconjugated protein molecules. Finally, the precipitates were resuspended with 40 μL of PBS (10 mM, pH 7.4) containing 5% sucrose, 1% BSA, 2% fructose, 0.4% Tween-20, and 1% PEG 20 000 and stored at 4 °C.

2.6. Fabrication of the Immunochromatographic Test Strip.

The QBs immunochromatographic test strip was composed of three main elements: a sample pad, a nitrocellulose (NC) membrane, and an absorbent pad. The hapten-OVA coating antigen and goat antimouse IgG were sprayed onto the NC membrane to form the test line and control line by the XYZ-3050 dispensing platform, respectively. Then the NC membrane was dried at 37 °C under vacuum for 2 h. The three components were sequentially attached to a polyvinyl chloride baking card with 2 mm overlaps. Finally, the assembled strip products were cut into the lateral flow test strip with 3.5 mm width and were fitted into plastic cassettes and stored with desiccants at room temperature.

2.7. Assay Procedure.

The developed QBs–FITSA for TEB detection relied on the competitive mechanism that TEB in the sample extract solutions competed with hapten–OVA on the NC membrane to bind with QBs–mAb conjugates, which had an emission fluorescence signal at 620 nm following excitation at 365 nm (Figure 2). For a typical QBs–FITSA, 75 μL of sample solution (10 mM PBS with 5% methanol, pH 8.0) containing different concentrations of TEB mixed with 0.6 μL of QBs–mAb probes were deposited onto the sample pad of the QBs–FITSA to initiate the capillary action. After 15 min, the fluorescence intensities of QBs–mAb probes on the test line and the control line of QBs–FITSA were recorded by the fluorescence immunoassay analyzer for quantitative analysis. Standard curves were constructed in the form of B/B_0 vs log *C*, where *B* and B_0 represent the fluorescence intensity ratio (FI_T/FI_C) of the sample containing serial concentration of TEB and sample without TEB. The fluorescence images also could be observed with naked eyes for qualitative and semiquantatitive analysis under an ultraviolet emitting light at 365 nm.

2.8. Method Validation via Spiked Samples.

Wheat, cabbage, and cucumber of TEB-free certified by LC/MS-MS were used for the method validation and recovery study. Briefly, 10 g of ground wheat, cabbage, or cucumber was completely homogenized and then added with TEB at concentrations of 0.02, 0.05, and 0.1 mg/kg, respectively. These samples were extracted with 20 mL of acetonitrile for 30 min on a vortex shaker and then centrifuged at 5000 × *g* for 10 min. A 5 mL amount of the supernatant was collected and dried by nitrogen and then redissolved with 5 mL of PBS

containing 5% methanol. The solution was further diluted 20-fold in PBS containing 5% methanol and then analyzed with the QBs–FITSA.

3. RESULTS AND DISCUSSION

3.1. Characterization of Hapten and Antigen.

The structure of hapten was supported by LC/MS and ^{1}HNMR. LC/MS (ESI) calcd for $C_{22}H_{33}N_3O_3$: 387.25. Found: *m/z* 388.3 [M^+ + H] (Figure S1A). ^{1}HNMR (DMSO) results confirm that the carboxyl group has successfully attached to TEB molecules (Figure S1B): δ 11.98 (s, 1H, −COOH), 8.51 (s, 1H, Ar–H), 8.01 (s, 1H, Ar–H), 6.99–7.06 (m, 4H, Ph-H), 4.52 (s, 1H, CH_2), 4.25–4.39 (m, 2H, CH_2), 2.47–2.54 (m, 2H, CH_2), 2.17–2.20 (t, 2H, CH_2), 1.89–1.90 (m, 1H, CH_2), 1.73–1.76 (m, 1H, CH_2), 1.62–1.64 (m, 1H, CH_2), 1.51–1.60 (s, 4H, $2CH_2$), 1.31–1.47 (m, 2H, CH_2), 1.29 (s, 9H, $3CH_3$).

BSA and OVA were used as carrier proteins to synthesize immunogen and coating antigen, respectively. From the UV–vis spectra of hapten, carrier protein, and conjugates, both immunogen (Hapten-BSA) and coating antigen (Hapten-OVA) conjugates show obvious absorption shifts compared to the hapten and carrier protein (Figure S2). The MALDI-TOF/TOF results show molar ratios of 33 and 24 for Hapten-OVA and Hapten-BSA, respectively (Figure S3).

3.2. Characterization of mAbs.

The incubation curve of absorbance value against TEB concentration by indirect competitive ELISA shows a linear range of detection (IC_{20}–IC_{80}) of 0.07–0.54 ng mL^{-1} and a half-maximal inhibitory concentration (IC_{50}) of 0.19 ng mL^{-1} (Figure 3A), which is 3 orders of magnitude more sensitive than reported in the previous study due in part to a longer length of the spacer arm and more appropriate active combining groups of hapten to favor the epitope exposure.[17–19] To the best of our knowledge, so far, our developed mAb shows the most sensitive antibodies for TEB detection among the reported methods.[19,20] The specificity test using 5 triazole analogues of TEB has a cross-reactivity (CR) value <0.01% (Table 1).

3.3. Characterization of QBs–mAb Conjugates.

The anti-TEB mAb-labeled QBs conjugates (QBs–mAb) were prepared using carbodiimide cross-linker chemistry by coupling the $-NH_2$ of antibody with the −COOH of the QBs. The QBs–mAb conjugates were characterized with dynamic light scattering (DLS) analysis. DLS analysis shows the hydrodynamic diameters of QBs–mAb increase from 142 to 190 nm, implying the successful attachment of the mAb to the surface of QBs (Figure 3B). The further immunochromatographic test strip assay shows that the mAb immobilized on the QBs surface with functional bioactivity. These results support the successful conjugation of the QBs–mAb.

3.4. Optimization of QBs–FITSA.

For a competitive immunoassay, the concentration of both antibody and competitor antigen, referred as the concentration of QBs–mAb and coating antigen (Hapten-OVA) in our QBs–

FITSA had a significant effect on the assay sensitivity. A “checkerboard titration” test was employed to optimize the concentration of QBs–mAb in running buffer and Hapten-OVA in test line. The inhibition rate (IR) and fluorescence intensity (FI) of both lines were chosen as the evaluation criteria, whose IR is calculated according to the formula: IR = $(1 - B/B_0) \times 100\%$, where B and B_0 represent the FI ratio (FI of test line/FI of control line) of the sample containing 0.2 ng mL^{-1} TEB and sample without TEB. As shown in Table S1, 0.6 mg mL^{-1} of Hapten-OVA and 0.6 μL of QBs–mAb give the highest inhibition rate and a strong fluorescence intensity of over 500 which is easy to evaluate with the naked eye.

The methanol content of the sample extract and pH value of running buffer on assay characteristics were also evaluated. Organic cosolvents are often used to improve TEB solubility. pH has a significant effect on the reaction of antibody and antigen pair. Following evaluation of many conditions, 5% methanol in buffer at a pH of 8.0 with the QBs–FITSA shows the best performance (data not shown).

3.5. Analytical Performance of QBs–FITSA.

TEB-standard solutions were diluted with running buffer (10 mM PBS with 5% methanol, pH 8.0) at various concentrations from 0.01 to 20 ng mL^{-1} and used to evaluate the sensitivity of the QBs–FITSA. The fluorescence intensities of the T line gradually decrease with increasing concentration of TEB, and the threshold concentration of TEB (or visual cutoff value: the color of T line completely disappeares) is 2.5 ng mL^{-1} (Figure 4A). Furthermore, the corresponding fluorescence intensities ratio of the test line and control line were calculated for quantitative analysis. As shown in Figure 4B, the calibration curve of the binding rate (B/B_0) exhibits a linear function of the concentration of TEB in the range from 0.02 to 1.25 ng mL^{-1} (IC_{10}–IC_{90}). The limit of detection (LOD) is calculated at 0.02 ng mL^{-1} (IC_{10}), which is 3 orders of magnitude lower than the maximum residue limit (MRL) of TEB residue (50 ng mL^{-1}) (Nos. EU 750/2010 and GB-2763–2016).[7,8]

The specificity of the QBs–FITSA was tested with other four analogues. As shown in Figure S4, the four analogues (1000 ng mL^{-1}) and blank control exhibit almost equal and obvious fluorescence signals of test line (negative signal), whereas 5 ng mL^{-1} TEB shows complete absence of fluorescence signal on the test line (positive signal), indicating the high specificity of the QBS-FITSA toward the target analyte. These results are consistent with the cross-reactivity results of ELISA.

In addition, compared to previously reported recognition element-based assays for TEB quantification, the developed QBS-FITSA shows superior performance in terms of both sensitivity and speed of analysis (Table S2).

3.6. Analysis of TEB in Spiked and Real Samples.

Different concentrations of TEB (0.02, 0.05, and 0.1 mg kg^{-1}) in spiked crops and vegetables samples (wheat, cabbage, and cucumber) were measured to evaluate the practicability of the QBs–FITSA. Table 2 summarizes the recovery results of the QBs–FITSA of spiked samples. The average recovery values ($n = 3$) range from 82% to 110%. The recovery results of the spiked samples obtained from QBs–FITSA are in good

agreement with those obtained from LC/MS-MS, confirming the reliability and practicability of this QBs–FITSA for TEB analysis.

We also analyzed TEB residues with the developed QBs–FITSA in 9 real samples (wheat, cabbag,e and cucumber) purchased from three local supermarket in Nanjing, Jiangsu, China. All residues are below the QBs–FITSA LOD level or not found in these samples (Figure S5). However, the corresponding spiked samples with 0.05 mg/kg TEB show the expected values (Figure 5). Thus, the QBs–FITSA can be a valuable analytical tool for the rapid and simple determination of TEB residues in processed crops and vegetables.

In this study, we developed a QBs–FITSA for quantitative determination of residues of TEB in agricultural products (wheat, cabbage, and cucumber) using a specific monoclonal antibody, which was screened based on a hapten of *p*-chloro-substituted derivatives of TEB. Under optimal conditions, the proposed QBs–FITSA could detect a minimum of 0.02–1.25 ng mL^{-1} TEB in 15 min. The recovery results of QBs–FITSA showed good agreement with the LC/MS-MS method for spiked sample, indicating the reliability and practicability of the developed assay. The QBs–FITSA showed superior performance in terms of both sensitivity and rapidity compared with previously reported assays for TEB. Therefore, with the advantages of low cost, simplicity, high sensitivity, and fast response, the proposed QBs–FITSA showed great promise for its implementation in TEB residue monitoring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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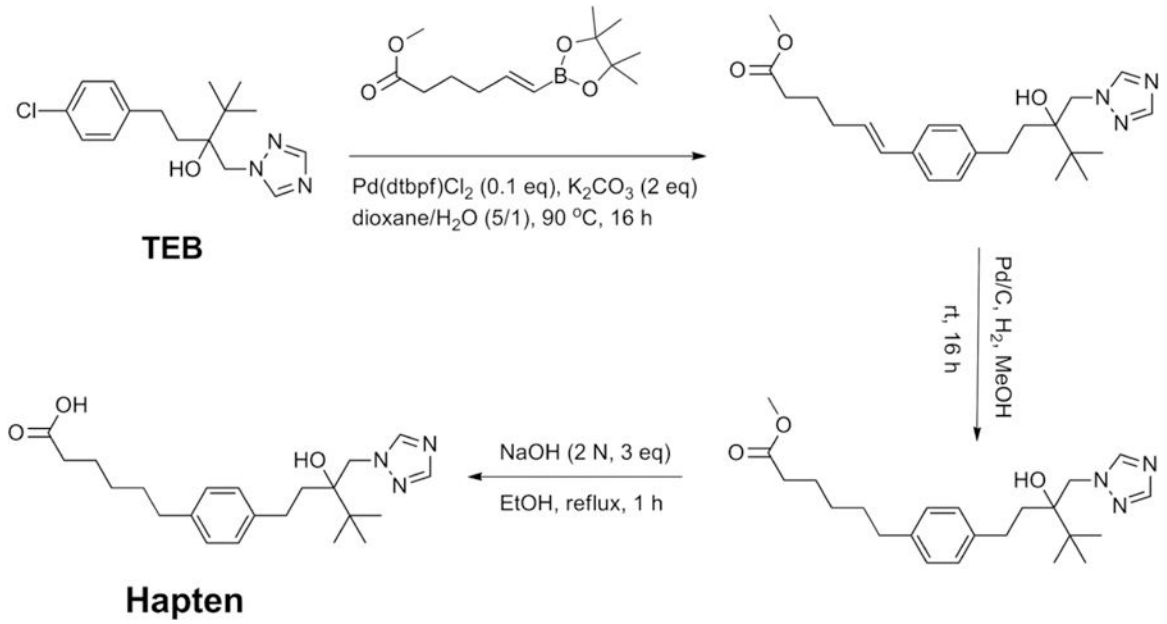


Figure 1.
Synthesis schematic and chemical structure of TEB and hapten.

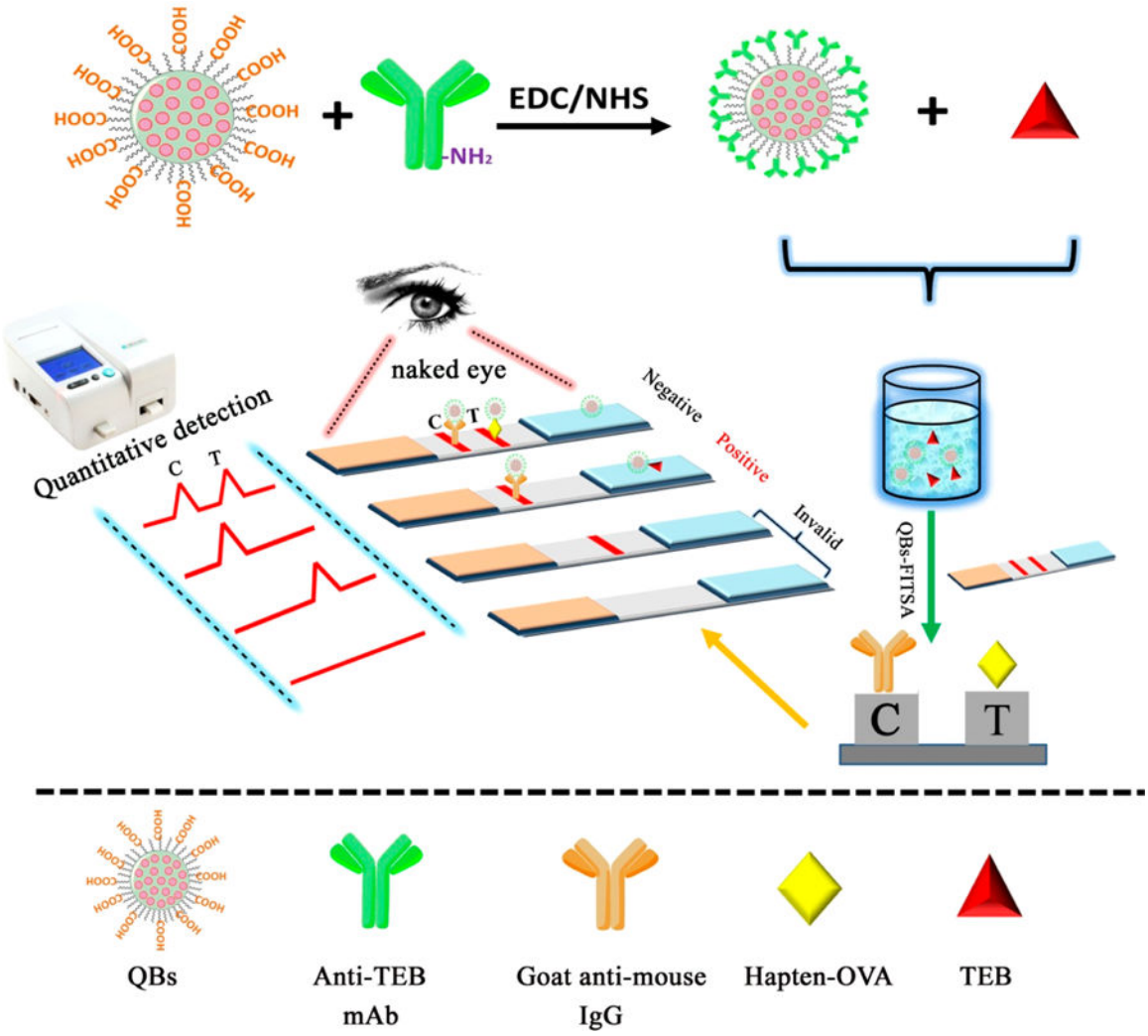


Figure 2.

Schematic illustration of the QBs–FITSA for quantitative detection of TEB using a novel anti-TEB monoclonal antibody (mAb) and high fluorescent quantum dot beads (QBs).

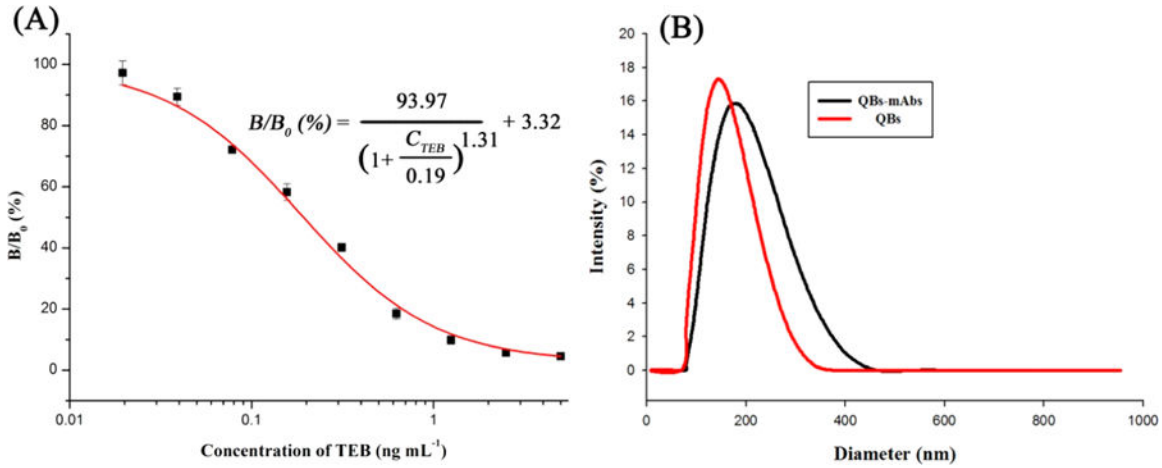


Figure 3.

(A) Standard curve of binding rate (B/B_0) against concentration of TEB by ELISA. (B) Hydrodynamic diameter of bare QBs and QBs–mAbs.

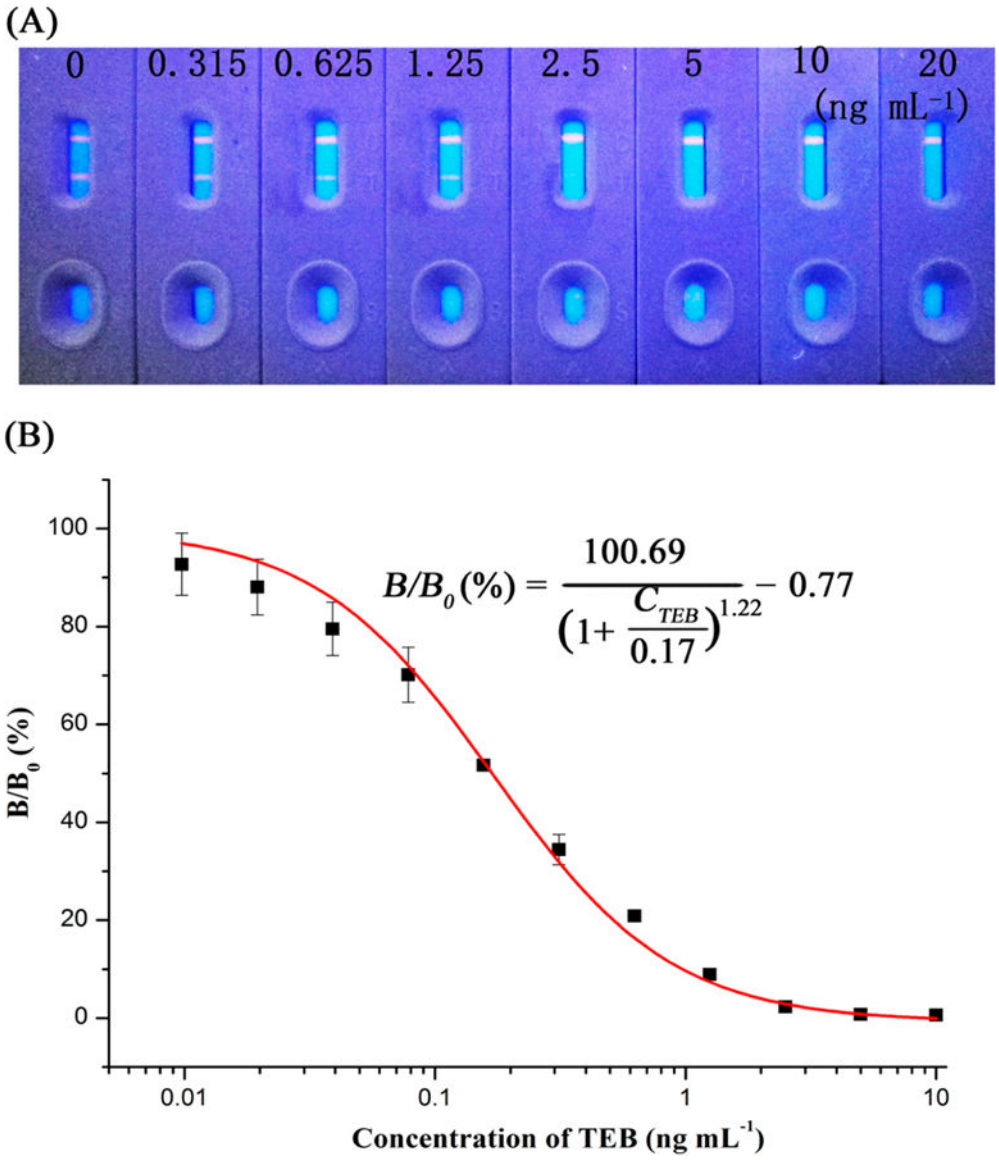


Figure 4.

(A) Photos of different concentration of TEB by QBs–FITSA. (B) Corresponding quantitative curve of binding rate (B/B_0) against concentration of TEB, where B and B_0 represent the fluorescence intensity ratio (FI_T/FI_C) of the sample containing TEB and sample without TEB.

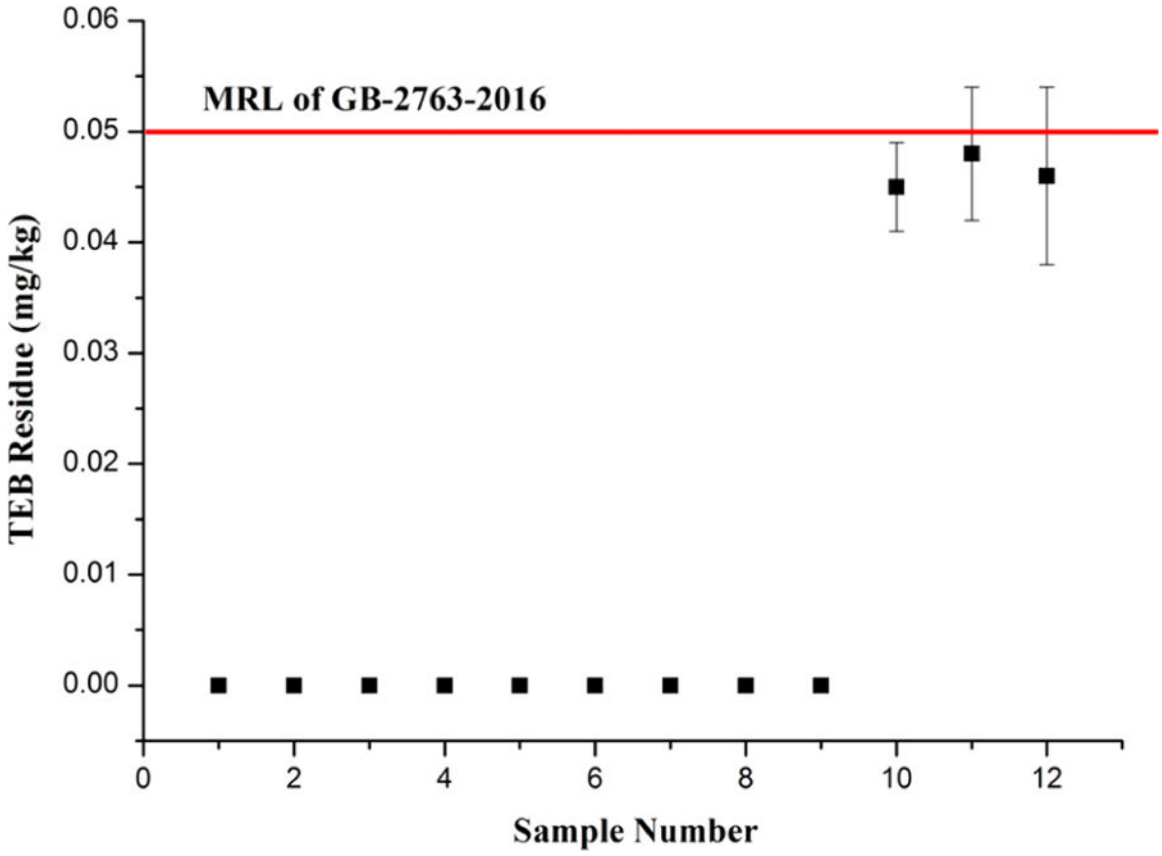


Figure 5.

Result of tebuconazole (TEB) detection in 9 real samples by QBs–FITSA, including wheat samples (1–3), cabbage samples (4–6), and cucumber samples (7–9). Samples 10, 11, and 12 represent samples 1, 4, and 7 spiked with 0.05 mg/kg TEB, respectively.

Table 1.

Selectivity Study of mAb Against TEB and Other Triazole Analogue Compounds by ELISA

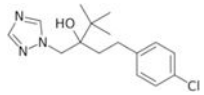
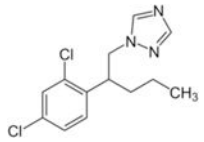
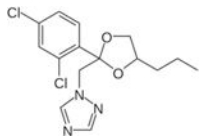
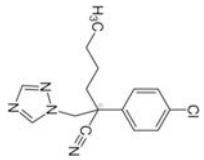
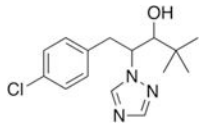
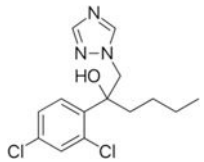
Compounds	Structure	IC_{50} (ng mL^{-1})	Cross-reactivity
Tebuconazole		0.19	100%
Penconazole		> 5,000	< 0.01%
Propiconazole		> 5,000	< 0.01%
Myclobutanil		> 5,000	< 0.01%
Paclobutrazol		> 5,000	< 0.01%
Hexaconazole		> 5,000	< 0.01%

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Table 2.

Average Recoveries of the QBs–FITSA and LC/MS-MS in TEB-Spiked Samples ($n = 3$)

		QBs–FITSA			LC/MS-MS		
samples	**spiked concentration (mg kg^{-1})**	**found (mg kg^{-1})**	**recovery (%)**	**CV[a] (%)**	**found (mg kg^{-1})**	**recovery (%)**	**CV[a] (%)**
wheat	0.02	0.018	90.0	14.4	0.017	85.0	1.0
	0.05	0.044	88.0	3.0	0.045	90.0	3.2
	0.1	0.082	82.0	0.8	0.097	97.0	3.1
cabbage	0.02	0.019	95.0	4.2	0.018	90.0	2.2
	0.05	0.045	90.0	5.7	0.050	100.0	5.5
	0.1	0.089	89.0	8.5	0.092	92.0	9.3
cucumber	0.02	0.022	110.0	3.1	0.021	105.0	1.1
	0.05	0.042	84.0	8.5	0.043	86.0	7.1
	0.1	0.093	93.0	5.3	0.095	95.0	5.2

[a]Coefficient of variation.