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A highly sensitive immunoassay for atrazine based on covalently linking the small molecule hapten to a urea–glutaraldehyde network on a polystyrene surface

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ABSTRACT

A new enzyme-linked immunosorbent assay (ELISA) for atrazine was developed based on covalent bonding of the small molecule hapten, 2-mercaptopropionic acid-4-ethylamino-6-isopropylamino-1,3,5-triazine (MPA–atrazine), to urea-glutaraldehyde (UGA)-treated microtiter plates. In this assay, the microtiter plate surface was treated with the UGA network to both introduce amino groups, which were used to cross-link with the hapten carboxylate groups, and efficiently prevent non-specific adsorption of antibodies, which successfully eliminated the time-consuming routine blocking step. Compared with HNO_3-H_2SO_4-APTES-hapten coated ELISA (modified with a HNO_3-H_2SO_4-APTES mixture and covalent-linked hapten) and conventional ELISA (coated with hapten–carrier protein conjugates), the novel ELISA format increased the sensitivity by approximately 3.5-fold and 7.5-fold, respectively, and saved 2.5 h and 34 h of coating hapten time, respectively. The method's 50% inhibition concentration for atrazine was 5.54 ng mL^{-1}, and the limit of detection was 0.16 ng mL^{-1} after optimization of reaction conditions. Furthermore, the ELISA was adapted for analysis of atrazine in corn, rice, and water samples, demonstrating recoveries of 90%–108%. Thus, the assay provides a convenient alternative to conventional, laborious immunoassays for routine supervision of residue detection in food and the environment.

1. Introduction

Atrazine is one of the most prevalent herbicides for combating weeds in corn, sugarcane, and sorghum crops due to its selectivity for broadleaf weeds and annual grasses [1,2]. However, large-scale use of atrazine has led to the contamination of foods and drinking water through various pathways, causing serious health risks [3]. The United States Environmental Protection Agency (USEPA) has reported that atrazine overexposure may induce critical diseases, such as low blood pressure, weight loss, muscle spasms, and adrenal gland damage [4–5]. Consequently, to limit human exposure, the European Food Safety Authority (EFSA) has recommended setting the maximum residue levels (MRLs) for all cereals at 0.05 mg kg^{-1}. In accordance with the WHO, atrazine concentrations in drinking water are limited to 2 μg L^{-1} [6–8].

The analytical techniques used to determine atrazine include liquid chromatography-tandem mass spectrometry [9–10], high-performance liquid chromatography [11], ultra-fast liquid chromatography [12], molecularly imprinted photonic crystals [13], bacterial biosensors [14], electrochemical immunosensors [15], molecular-imprinted quartz crystal microbalance (QCM) sensors [16], enzyme-based biosensors [17], and polyaniline-based sensors [18]. However, these methods require expensive instrumentation and time-consuming sample pretreatments, resulting in complex and laborious screening procedures. As an alternative, the enzyme-linked immunosorbent assay (ELISA) has gained acceptance as a suitable technique because it is inexpensive, rapid, sensitive, and selective [19].

In traditional ELISAs, the small molecule hapten and carrier protein conjugates as coating antigens were fixed on the surface of microtiter plates through hydrophobic interactions [20–22]. However, such ELISAs exhibit several disadvantages. First, the ratio of hapten molecules to carrier protein is inconsistent and non-reproducible during conjugate preparation; thus, the assay standardization and evaluation of hapten–protein stoichiometry is unfavorable. Second, immobilization of hapten–protein conjugates on the surface relies on hydrophobic interactions, which often modifies the antigenicity owing to unsuitable presentation and orientation of the hapten molecules [23].

The direct immobilization of hapten molecules on a polystyrene (PS) support is a promising approach to avoid the drawbacks of traditional ELISA. Based on this principle, Feng et al. and Kaur et al. utilized a mixture of HNO_3-H_2SO_4 and 3-aminopropyltriethoxysilane (APTES) to generate amino groups on microtiter plate surface for covalent linkage of hapten molecules, and corresponding immunoassays were successfully developed for the detection of bisphenol and 2,4-dichlorophenoxyacetic acid [24,25]. However, these methods are not suitable for practical

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applications because the HNO_3-H_2SO_4 mixture presents a risk for operators. In addition, the density of anchor groups on the treated surface is often too high, which results in a high coupling density and decreased sensitivity.

Herein, we described a facile and environmentally friendly processing method for generating amino groups on PS microtiter wells by applying a urea-glutaraldehyde (UGA) mixture that is used as an adhesive agent in the furniture industry. This method allowed us to covalently link 2-mercaptopropionic acid-4-ethylamino-6-isopropylamino-1,3,5-triazine (MPA-atrazine) to develop a highly sensitive immunoassay (UGA-hapten coated ELISA) for the detection of atrazine. In addition, we developed two immunoassays for comparison, respectively employing HNO_3-H_2SO_4 and APTES as PS surface modifiers to covalently link the MPA-atrazine (HNO_3-H_2SO_4-APTES-hapten coated ELISA) and atrazine-ovalbumin (OVA) conjugates as the coating antigen (conventional ELISA) (Fig. 1). The analytical performances of the developed assays in food/water matrices were further established by performing spike and recovery studies with corn, rice, and drinking water samples.

2. Materials and methods

2.1. Materials and instruments

Atrazine, simazine, melamine, chlorpyrifos, monocrotophos, and parathion were obtained from Three New Science and Technology Chemical Co., Ltd. (Shandong, China). Bovine serum albumin (BSA), OVA, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), *N*,*N*-Dimethylformamide (DMF) and APTES were purchased from Sigma-Aldrich (St. Louis, MO, USA). The monoclonal antibody against atrazine and goat anti-mouse IgG- horse radish peroxidase (HRP) were obtained from Beijing LEYBOLD Cable Technology Co. Ltd. (Beijing, China). Other chemicals were obtained from Tianjin Chemicals Inc. (Tianjin, China). All chemicals, reagents, and solvents used in this study were of high-purity analytical grade. Buffers were made using Milli-Q water (Millipore, Bedford, MA, USA). By dissolving a calculated amount of urea in a respective amount of aqueous glutaraldehyde (GA) solution, the raw UGA mix was prepared. Mixing was kept at room temperature until complete dissolution of urea, so the procedure required no typical and laborious resin synthesis. The 96-well PS microtiter plates were obtained from Costar Inc. (Milpitas, CA, USA). A Multiskan MK3 ELISA reader (Thermo, USA) was applied to measure absorbance.

2.2. MPA-atrazine preparation

MPA-atrazine was synthesized as described previously [26]. Atrazine (1.3 g) and ethanol (60 mL) were mixed in a 150-mL flask. Then, KOH (2.4 g) and 3-mercaptopropionic acid (0.9 mL) were slowly added, and the resulting reaction mixture was heated at reflux for 3 h. After the reaction was completed, the solvent was removed by distillation under reduced pressure. The residue was completely dissolved in $NaHCO_3$ solution (5%) and extracted with chloroform (10 mL) three times. After dehydrating the reaction mixture with Na_2SO_4, the solvent was concentrated under reduced pressure to yield the targeted product (MPA-atrazine). The result was monitored by thin-layer chromatography (TLC) and ^1H nuclear magnetic resonance (NMR) spectroscopy.

2.3. Modification of microtiter plates using UGA and directly coating MPA-atrazine

Microtiter plates were incubated with a UGA mix with the proper ratio at 37 °C for 2 h. After washing the plates two times with deionized

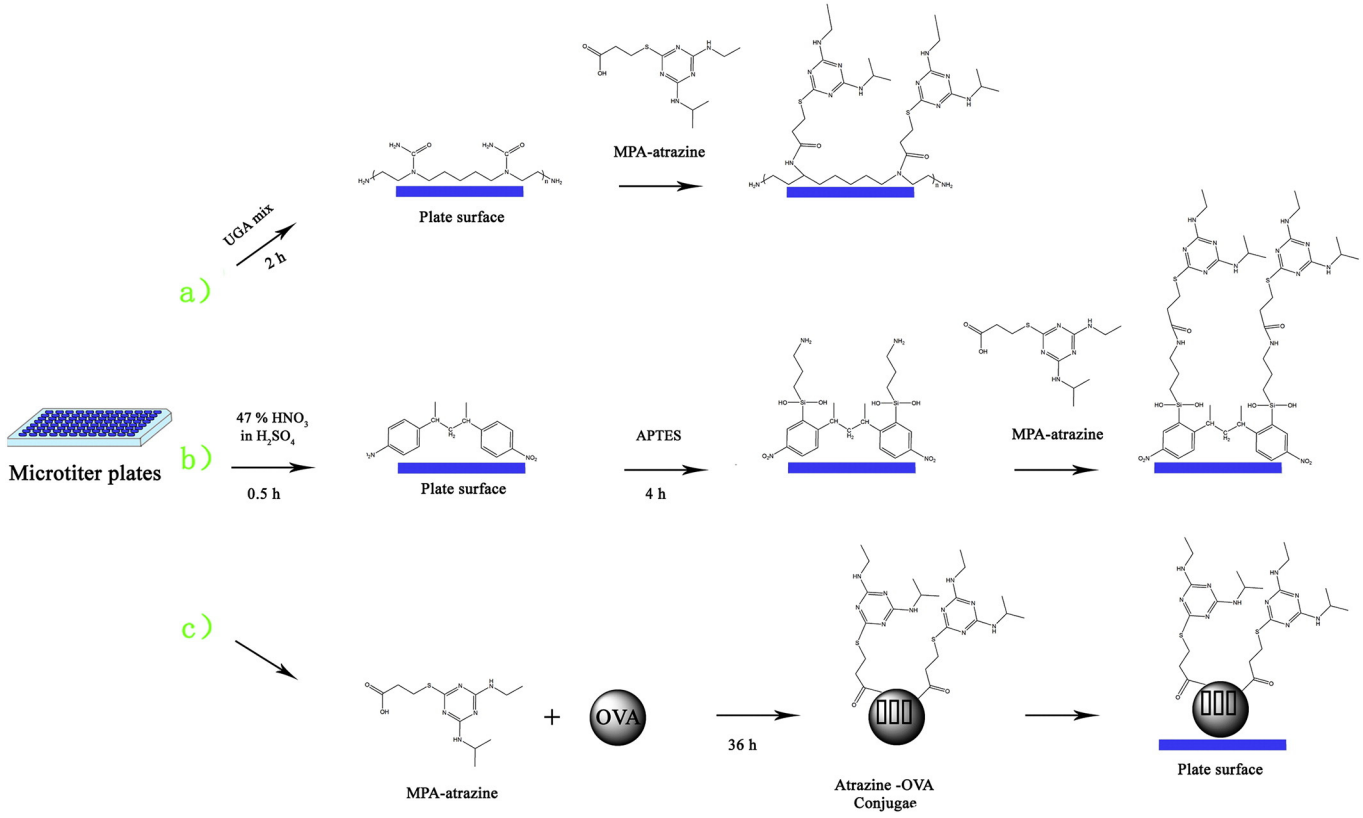


Fig. 1. Reaction mechanism of three different ELISA formats: a) UGA-hapten coated ELISA. The UGA was used to treat the microtiter plate surface for 2 h to introduce amino groups that can covalent link MPA–atrazine; b) HNO_3-H_2SO_4-APTES-hapten coated ELISA. The microtiter plate surface was treated with HNO_3-H_2SO_4 mixture for 0.5 h to create nitro groups, and then was reacted with 5% APTES for 4 h to generate amino groups that can covalently attach MPA-atrazine; c) Conventional ELISA. Atrazine-OVA conjugate was synthesized with MPA- atrazine and OVA spending 36 h, and coated the microtiter plate surface by the physical adsorption capacity.

water, amino groups of microtiter plates were created. Activated MPA-atrazine (100 μL, 175 nmol mL^{-1}) was added to each modified well and incubated for 12 h at 4 °C.

2.4. Modification of microtiter plates using the HNO_3-H_2SO_4 and APTES mixture and directly coating MPA-atrazine

Microtiter plates were first a strong acid treated with a HNO_3-H_2SO_4 mixture (47:53, *v*/v) for 30 min at room temperature. After washing thoroughly three times with deionized water, nitro groups were introduced on the PS surface. The modified wells were then incubated with 5% APTES aqueous solution (pH 5.5) at room temperature for 2 h and then reacted at 62 °C for 2 h to generate amino groups on the PS surface. Activated MPA-atrazine (100 μL, 350 nmol mL^{-1}) was then added to each modified well and incubated overnight at 4 °C. Blocking was done with formaldehyde solution (100 μL, 0.2 mol L^{-1}) by incubating the plates for 2 h at 37 °C to block the unbound sites of the PS surface.

2.5. Atrazine-OVA conjugate preparation and coating in the microtiter plates

Atrazine-protein conjugates were synthesized using the DCC method. Briefly, MPA-atrazine (10 mg), NHS (6 mg), and DCC (12 mg) were dissolved in DMF (2 mL) for 12 h at room temperature. Next, the mixture was centrifuged and the supernatant collected. To this solution, 10 mg mL^{-1} OVA in borate buffer was added, and the mixture was stirred at room temperature for 24 h. The atrazine-OVA conjugate was then purified by dialysis in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). The atrazine-OVA conjugates were identified by UV–vis spectrophotometry. The microtiter plate wells were coated with 100 μL atrazine-protein conjugates in coating buffer for 12 h at 4 °C. To solve the non-specific adsorption problem, plates were treated with blocking buffer involving 1% BSA at 37 °C for 2 h and washed three times with PBST (0.01 mol L^{-1}, pH 7.4, PBS with 0.05% tween 20) for further reaction.

2.6. Competitive inhibition ELISA protocol

The three kinds of hapten-coated plates prepared were each used in the following steps. Optimized specific anti-atrazine antibody (50 μL) and different concentrations of free atrazine (50 μL) were added to each well. After incubating 1.5 h at 37 °C, the plates were washed thoroughly with PBST, and then 100 μL secondary antibody (goat anti-mouse IgG-HRP) at proper dilution (1:3000) was added for 1 h at 37 °C. The tetramethylbenzidine (TMB) substrate solution (100 μL $well^{-1}$) was added after a final washing step, and the enzymatic reaction was incubated for 15 min at room temperature and stopped by the addition of 2 mol L^{-1} H_2SO_4 (50 μL $well^{-1}$). The absorbance of each well was measured at 450 nm using an ELISA reader.

2.7. Sensitivity and specificity

The sensitivity of the immunoassays was evaluated using normalized assay signals as $\%B/B_0$ based on the following formula:

$$\frac{B}{B_0} = \frac{A - A_{ex}}{A_0 - A_{ex}} \times 100$$

where A is the absorbance of hapten at the standard concentration, A_0 is the absorbance at zero hapten concentration, and A_{ex} is the absorbance at excessive hapten concentration.

The specificity of the immunoassay was characterized by cross-reactivity (CR). A standard solution of each compound was dissolved in methanol and diluted in PBS. Then, CR was determined for five different compounds structurally related to atrazine by performing competitive assays using the 50% inhibition concentration (IC_{50}) value and calculated as

$$\mathrm{CR}\ (\%) = \frac{IC_{50}\ \text{for atrazine}}{IC_{50}\ \text{for analogues}} \times 100$$

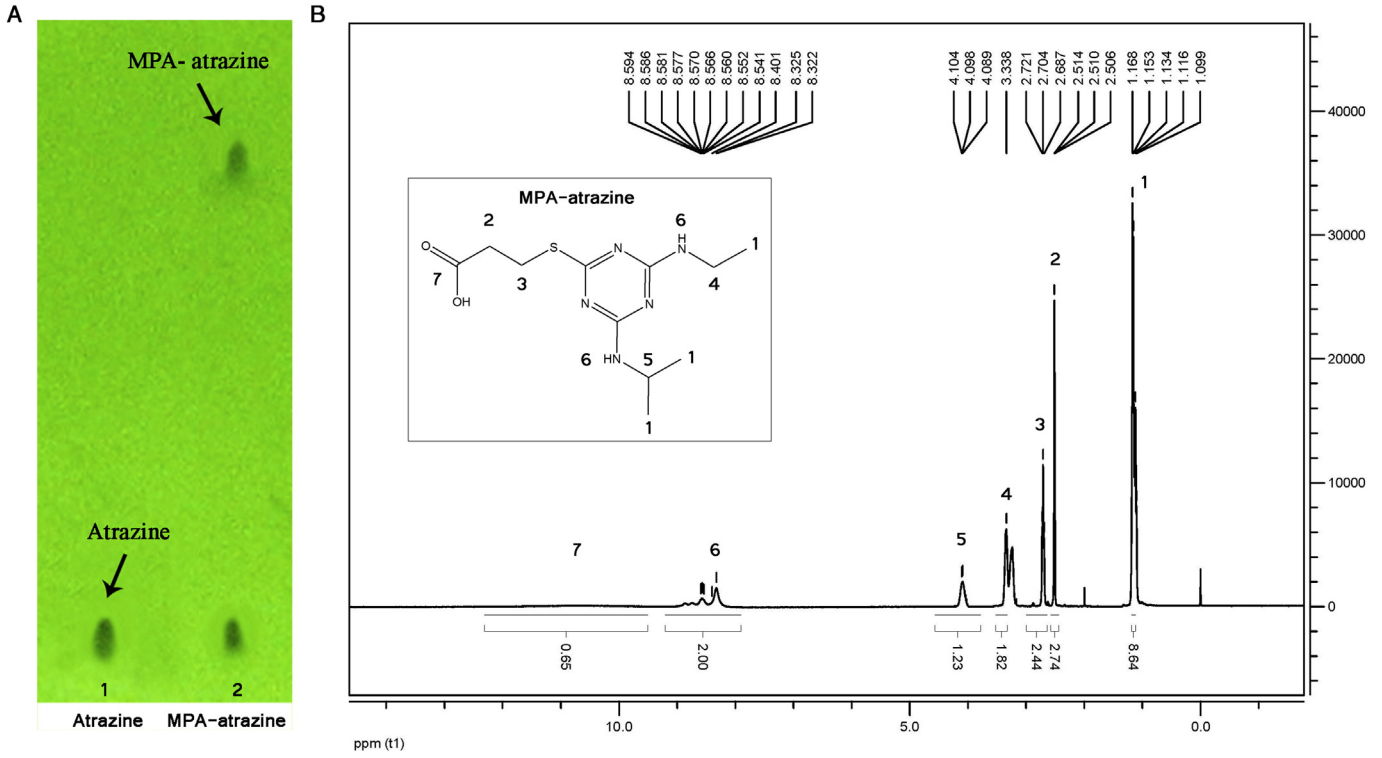


Fig. 2. The verification of MPA-atrazine: a) TLC of MPA-atrazine. Compared with atrazine original drug, there was new product (MPA-atrazine) generated; b) ^1H HMR spectrum of MPA-atrazine. The inset showed details of the fine peaks corresponding to some of MPA-atrazine.

2.8. Sample preparation

For real samples, matrix effects are a common challenge in immunoassays. Two different cereal samples (corn and rice) and drinking water were chosen to evaluate the performance of the immunoassays. Rice and corn were purchased from the local market in Tianjin. The crushed corn and rice samples (20 g) were respectively mixed with 20 mL methanol/water (1:1, v/v) and then shaken for 30 min. After centrifuging at 4000 × *g* for 30 min, the supernatants were transferred to a 25-mL volumetric flask. After adding sodium chloride (5 mol L^{-1}, 5 mL), the sample was extracted with a methylene chloride and petroleum ether mixture (3.5:6.5, v/v). The supernatant was evaporated, and the residue was dissolved in 0.5 mL methanol and 0.01 mol L^{-1} PBS. Finally, the corn and rice samples were spiked with a series of atrazine standards (5, 10, and 50 μg kg^{-1}) for immunoassay analysis. The drinking water was also spiked with known concentrations of atrazine (5, 10, and 50 μg kg^{-1}) and then analyzed by the ELISAs.

2.9. Statistical data analysis

Competitive curves of ELISAs were obtained by plotting the inhibition rate against the logarithm of atrazine concentration. Sigmoidal curves were fitted to four-parameter logistic equation, using the Origin pro 7.5 software package. The obtained results were expressed as mean ± standard deviations (SD) and coefficient of variation (CV). Analysis of ELISA data and statistical analyses were carried out in Microsoft Excel 2007.

3. Results and discussion

3.1. MPA-atrazine and atrazine-OVA conjugates verification

The obtained MPA-atrazine was first verified by TLC. Fig. 2a showed that a new product was generated; thus, MPA-atrazine preparation was initially confirmed. The obtained MPA-atrazine was further identified from its ^{1}H HMR spectra (Fig. 2b). The ^{1}H NMR peaks (DMSO + D_2O) were observed at δ = 1.168–1.099 (m, CH_3, 9H), 2.514–2.510 (t, SCH_2CH_2COOH, 2H), 2.721–2.687 (t, SCH_2CH_2COOH, 2H), 3.338 (m, $NHCH_2CH_3$, 2H), 4.104–4.089 (t, $NHCH(CH_3)_2$, 1H), 8594–8.322 (m, $NHCH(CH_3)_2$, 2H), and 11.5 (s, COOH, 1H). The results indicated that MPA-atrazine was successfully synthesized.

Fig. 3 displayed marked differences in the UV spectra of equal concentrations of atrazine, OVA, and the atrazine-OVA conjugate, probably because the different substances (atrazine, atrazine-OVA conjugate, and OVA) have different absorbance ε. Thus, different absorbances in UV spectra were observed at same concentration. This confirmed that the atrazine-OVA conjugates were successfully prepared.

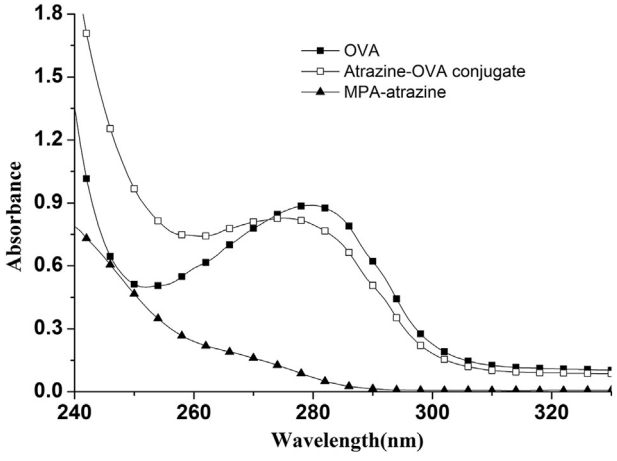


Fig. 3. UV spectrograms of MPA-atrazine (maximum absorption peak wavelength in 258.3 nm), atrazine-OVA conjugate (maximum absorption peak wavelength in 273 nm), and OVA (maximum absorption peak wavelength in 279.8 nm).

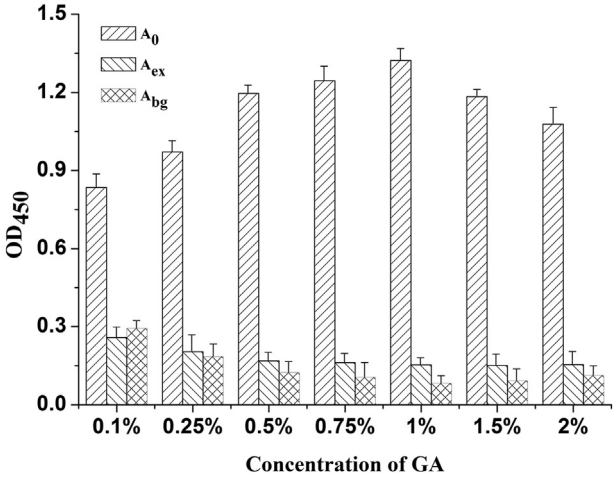


Fig. 4. Effect of the concentration of GA (from 0.1% to 2%) on A_0, A_{ex} and A_{bg} values. A_0 value is the absorbance at zero hapten concentration; A_{ex} value means the absorbance at excess hapten concentration; A_{bg} value is the absorbance at negative group. Each point represents the mean ± SD (standard deviation, n = 3).

3.2. Optimization of the essential steps of the assay

3.2.1. Effect of urea concentration and dilution level of GA

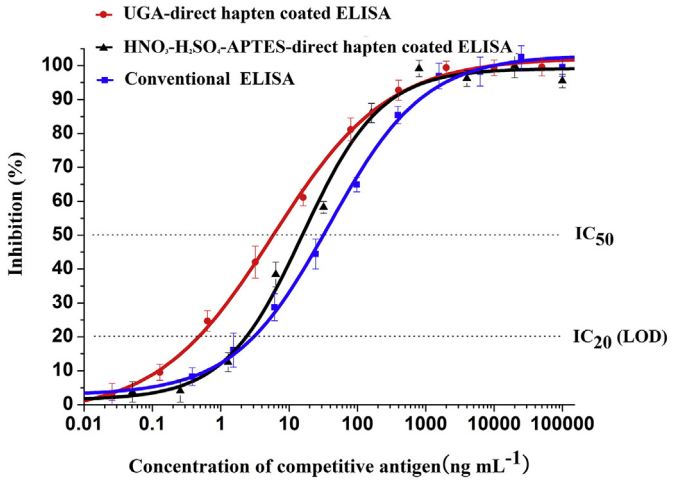
To directly immobilize MPA-atrazine, microtiter plates were pretreated with UGA. It has been suggested that a UGA network with amino groups was created and bound to the surface during this precoating step. Thereafter, the MPA-atrazine was linked to the surface-immobilized UGA at pH 8.0 by formation of resonance-stabilized imino bonds. Thus, the urea/GA molar ratio was optimized to obtain a stable network with amino groups.

The values of A_0 and A_{ex} were close to the background value (A_{bg}) for plates that were not treated with UGA (negative control), indicating that the MPA-atrazine was not immobilized. Fig. 4 showed the effect of different GA dilutions on the immunoassay parameters. When GA was diluted to less than 1%, the A_{bg} value decreased; the A_0 value increased with increasing GA concentration and started to decrease. The phenomenon was explained as follows. From 0.1% to 1% GA, the GA concentration increase would make the UGA network complex and react with more urea molecules to produce more amino groups. This complex network structure could effectively prevent non-specific adsorption of antibodies, thus causing the A_{bg} decrease. In particular, when using 1% GA, the A_{bg} value was only 0.6, which would allow elimination of the routine blocking step. On the other hand, at GA concentrations above 1%, the excess GA further increased and reacted with not only more urea but also the amino groups on the network itself. Thus, the number of free amino groups decreased, resulting in a decrease of the positive value (A_0). As a result, the maximum difference $A_0 - A_{ex}$ and lowest

Table 1
Influence of concentration of urea (from 0.2 to 1.6 mol L^{-1}) on assay parameters of UGA-hapten coated ELISA (n = 3).

Concentration (mol L^{-1})	Immunoassay parameters		
	A^a_0 Measured ± SD	A^b_{ex} Measured ± SD	A^c_{bg} Measured ± SD
0.2	1.213 ± 0.043	0.148 ± 0.012	0.079 ± 0.006
0.4	1.285 ± 0.028	0.151 ± 0.009	0.089 ± 0.011
0.8	1.322 ± 0.026	0.153 ± 0.011	0.083 ± 0.007
1.6	1.241 ± 0.050	0.193 ± 0.012	0.133 ± 0.009

[a] Absorbance at zero hapten concentration (0 μg ml^{-1}).
[b] Absorbance at excess hapten concentration (100 μg ml^{-1}).
[c] Absorbance at negative group.



ELISA formats	UGA-hapten coated ELISA	HNO_3-H_2SO_4-APTES-hapten coated ELISA	Conventional ELISA
Equation	$y=107.892-113.035/(1+(x/6.485)^{0.338})$	$y=112.089-107.253/(1+(x/27.856)^{0.477})$	$y=117.120-114.183/(1+(x/68.639)^{0.502})$
R^2	0.999	0.994	0.998
IC_{20}	0.16 ng mL^{-1}	0.63 ng mL^{-1}	1.2 ng mL^{-1}
IC_{50}	5.54 ng mL^{-1}	14.62 ng mL^{-1}	28.7 ng mL^{-1}

Fig. 5. The indirect competitive inhibition standard curves of the UGA-hapten coated ELISA, HNO_3-H_2SO_4-APTES-hapten coated ELISA and conventional ELISA. The LOD was calculated as IC_{20} value. Their LOD were 0.16 ng mL^{-1}, 0.63 ng mL^{-1}, and 1.20 ng mL^{-1}, respectively. Each point represents the mean ± SD (n = 3).

A_{bg} value were achieved at 1% GA (Fig. 4) and 0.8 mol L^{-1} urea (Table 1), these concentrations were used for subsequent experiments.

3.2.2. Effect of solid immobilization conditions

Assay sensitivity depends greatly on the solid phase. An ideal solid phase should be consistent, stable, and have a low background signal. The special ELISA microtiter plates (medium affinity) were selected because they can prevent non-specific adsorption.

In the general process of immunoassays, including HNO_3-H_2SO_4-APTES-hapten coated ELISA and conventional ELISA, the blocking step is necessary to prevent the nonspecific adsorption of antibodies. Thus, in UGA-hapten coated ELISA, we tested the blocking effectiveness of different blocking reagents such as formaldehyde, acetaldehyde, and ethanedial. However, the non-specific adsorption showed no obvious differences with or without the blocking step (data not shown). This might be because the blocking effect of the UGA network already prevented most non-specific adsorption (Fig. 4). Thus, using UGA as a modifier can both create amino groups on the microtiter plate surface and save at least 2 h by eliminating the blocking process.

3.3. Immunoassay quality assessment

3.3.1. Sensitivity

The standard curves of the three ELISA formats were showed in Fig. 5. The IC_{50} values of UGA-hapten coated ELISA, HNO_3-H_2SO_4-APTES-hapten coated ELISA, and conventional ELISA were about 5.54 ng mL^{-1}, 14.62 ng mL^{-1}, 28.70 ng mL^{-1}, respectively. Their limits of detection (LOD, calculated as IC_{20} value) were approximately 0.16 ng mL^{-1}, 0.63 ng mL^{-1}, and 1.20 ng mL^{-1}, respectively. Based on the LOD, the sensitivity of UGA-hapten coated ELISA was improved 7.5-folds approximately compared with that of conventional ELISA. This phenomenon was explained as follows. Because of the very low molecular weight and single reactive site of atrazine hapten, the reactive sites were easily screened by OVA in the atrazine-OVA conjugate. The coating of the atrazine-OVA conjugate on the surface of microtiter plates depends on hydrophobic interactions, which might involve significant conformational changes that allowed large OVA molecules to easily screen atrazine molecules during the coating process. In contrast, the atrazine was directly covalently linked with the surface of microtiter plates in the UGA-hapten coated ELISA. Thus, this format avoided the screening effect of the carrier protein and generated a UGA network on the surface to increase the distance between atrazine molecules and surface, thereby exposing more reactive sites.

Although they both employed hapten covalently linked to the surface, the UGA-hapten coated ELISA displayed about 3.9-fold higher sensitivity than the HNO_3-H_2SO_4-APTES-hapten coated ELISA. This high sensitivity would be caused by a decrease in non-specific adsorption. In the UGA-hapten coated ELISA, the UGA network can efficiently prevent non-specific adsorption of antibodies. However, in HNO_3-H_2SO_4-

Table 2
CRs of the inhibition assay with selected compounds included atrazine, simazin, melamine, chlorpyrifos, monocrotophos and parathion (n = 3).

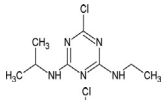
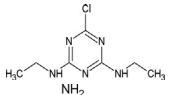
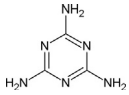
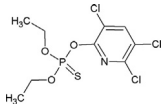
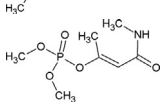
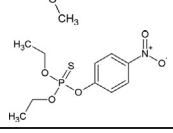
Compounds	Structure	IC_{50} (μg mL^{-1})	%CR
Atrazine		0.006	100
Simazine		0.061	19
Melamine		30.3	0.043
Chlorpyrifos		1.12×10^4	0.00012
Monocrotophos		1.78×10^6	7.29×10^{-7}
Parathion		165.30	0.0079

Table 3
Accuracy and precision of atrazine in spiked samples (water, rice and corn) by ELISAs based on intra-assays.

ELISA formats		Sample (μg kg^{-1})								
		Water			Rice			Corn		
		5	10	50	5	10	50	5	10	50
UGA-hapten coated ELISA	Measured (μg kg^{-1})	4.5 ± 0.15	9.3 ± 0.25	48.5 ± 0.51	5.4 ± 0.24	9.3 ± 0.30	49.0 ± 0.99	4.6 ± 0.16	9.3 ± 0.24	50.3 ± 1.28
	Recovery (%)	90.1	92.5	97.1	108.1	92.8	98.2	92.0	93.2	100.5
	CV (%)	3.26	2.65	1.06	4.47	3.21	2.03	3.58	2.6	2.54
HNO_3-H_2SO_4-APTES-hapten coated ELISA	Measured (μg kg^{-1})	5.4 ± 0.20	8.9 ± 0.14	53.1 ± 1.93	4.3 ± 0.11	11.6 ± 0.32	49.4 ± 0.74	5 ± 0.14	9.6 ± 0.32	49.2 ± 1.22
	Recovery (%)	108.0	89.8	106.2	86.4	116.2	98.8	100.5	96.4	98.5
	CV (%)	3.71	1.58	3.64	2.59	2.78	1.5	2.77	3.29	2.48
Conventional ELISA	Measured (μg kg^{-1})	4.3 ± 0.16	9.7 ± 0.31	51.6 ± 1.52	4.3 ± 0.08	9.2 ± 0.19	50.3 ± 1.59	4.6 ± 0.16	9.2 ± 0.20	51.3 ± 1.55
	Recovery (%)	86.3	97.8	103.2	86.6	92.4	100.6	92.3	92.7	102.6
	CV (%)	3.8	3.21	2.95	1.88	2.04	3.17	3.53	2.12	3.03

Intra-assay variation was determined by five replicates on a single day.

APTES-hapten coated ELISA, the carbon chain and benzene ring substituents cannot efficiently prevent non-specific adsorption can easily couple in high density, thus yielding lower sensitivity.

3.3.2. Specificity and CR of the anti-atrazine antibody

CR is used as an important parameter for evaluating the specificity of immunoassays. Table 2 showed the sensitivity and CR values of anti-atrazine antibodies with atrazine and its analogues. The UGA-hapten coated ELISA showed very high sensitivity and specificity against the target molecule atrazine (IC_{50}: 0.006 μg mL^{-1}) versus similarly structured atrazine analogues such as simazine (IC_{50}: 0.061 μg mL^{-1}) and melamine (IC_{50}: 30.3 μg mL^{-1}) and structurally unrelated compounds such as chlorpyrifos (IC_{50}: 1.12 × 10^4 μg mL^{-1}), monocrotophos (IC_{50}: 1.78 × 10^6 μg mL^{-1}), and parathion (IC_{50}: 165.30 μg mL^{-1}). This assay had CR of 100% to atrazine, 19% to simazine, and less than 0.1% to the other compounds. The results were similar to those observed with the other two ELISA formats. Thus, the anti-atrazine antibody was very specific for atrazine.

3.3.3. Recovery

Corn, rice, and water samples were spiked with atrazine at 5, 10, 50 μg kg^{-1} and then analyzed with the UGA-hapten coated ELISA, HNO_3-H_2SO_4-APTES-hapten coated ELISA and conventional ELISA. Each sample was evaluated five times in duplicate and on five different days to verify the repeatability.

As shown in Table 3 and Table 4, the intra-assay recoveries ranged from 90.1%–108.1% with CVs of 1.06%–3.58% and the inter-assay recoveries were in the range of 91.5%–114.3% with CVs of 3.52%–8.34% in UGA-hapten coated ELISA. These recoveries were consistent with those in the other ELISA formats, but the CVs were much lower than those of the other ELISA formats. Thus, the results indicate that the UGA-hapten coated ELISA exhibited higher stability and successfully detected atrazine in the real samples tested.

The higher stability of UGA-hapten coated ELISA might be explained as follows. In conventional ELISA, the covalent bond of the atrazine-OVA conjugate can dissociate during storage, and the ratio of atrazine and OVA molecules is inconsistent and non-reproducible during conjugate formation. In the HNO_3-H_2SO_4-APTES-hapten coated ELISA, the PS surface was easily burned and made uneven by treatment with strong acid (HNO_3 and H_2SO_4), and the reaction was non-reproducible. However, UGA-hapten coated ELISA eliminated the complex preparation of atrazine-OVA conjugate and the use of strong acid, thus avoiding the previous drawbacks.

In summary, the operating procedures and the detection performances of the UGA-hapten coated ELISA were superior to those of HNO_3-H_2SO_4-APTES-hapten coated ELISA and conventional ELISA. In UGA-hapten coated ELISA, the hapten-coating process consumed only 2 h, which was shorter than the HNO_3-H_2SO_4-APTES-hapten coated ELISA (4.5 h) and the conventional ELISA (36 h). Furthermore, although there was no blocking step, the UGA-hapten coated ELISA exhibited improved sensitivity (LOD = 0.16 ng mL^{-1}) and stability compared to the other two ELISA formats.

4. Conclusion

We have developed a new strategy for the highly sensitive detection of atrazine by employing small molecules hapten covalently linked to UGA-treated microtiter plates. Compared with the other two ELISA formats, the novel method was simpler and shorter features while exhibiting high sensitivity, high stability, and high selectivity. The transferability of the findings and the parameters defined and verified for the immunoassays to other analytes needs to be confirmed. This is the focus of ongoing research.

Table 4
Accuracy and precision of atrazine in spiked samples (water, rice and corn) by ELISAs based on inter-assays.

ELISA formats		Sample (μg kg^{-1})								
		Water			Rice			Corn		
		5	10	50	5	10	50	5	10	50
UGA-hapten coated ELISA	Measured (μg kg^{-1})	4.8 ± 0.25	9.1 ± 0.32	49.2 ± 3.09	5.7 ± 0.29	9 ± 0.75	52.5 ± 2.01	5.1 ± 0.42	9.2 ± 0.41	50.5 ± 3.41
	Recovery (%)	96.2	91.5	98.4	114.3	90.7	105.4	102.2	92.4	101.7
	CV (%)	5.21	3.52	6.29	5.13	8.34	3.82	8.24	4.41	6.76
HNO_3-H_2SO_4-APTES-hapten coated ELISA	Measured (μg kg^{-1})	5.5 ± 0.27	8.8 ± 0.81	54.6 ± 6.15	4.5 ± 0.34	11.2 ± 0.55	49.7 ± 1.85	5.2 ± 0.52	9.3 ± 0.66	49.8 ± 3.15
	Recovery (%)	110.2	88.7	109.2	90.3	113.1	99.4	104.4	93.9	99.6
	CV (%)	4.87	9.21	11.26	7.62	4.89	3.73	10.02	7.11	6.32
Conventional ELISA	Measured(μg kg^{-1})	4.1 ± 0.31	9.1 ± 0.60	52.8 ± 1.84	4.1 ± 0.21	9.8 ± 0.94	52.6 ± 4.53	4.9 ± 0.23	8.8 ± 0.62	52.5 ± 3.74
	Recovery (%)	82.5	91.4	105.6	82.8	98.5	105.2	98.1	88.9	105.3
	CV (%)	7.57	6.55	3.48	5.16	9.63	8.61	4.66	7.07	7.14

Inter-assay variation was determined by five replicates in five different days.

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