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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

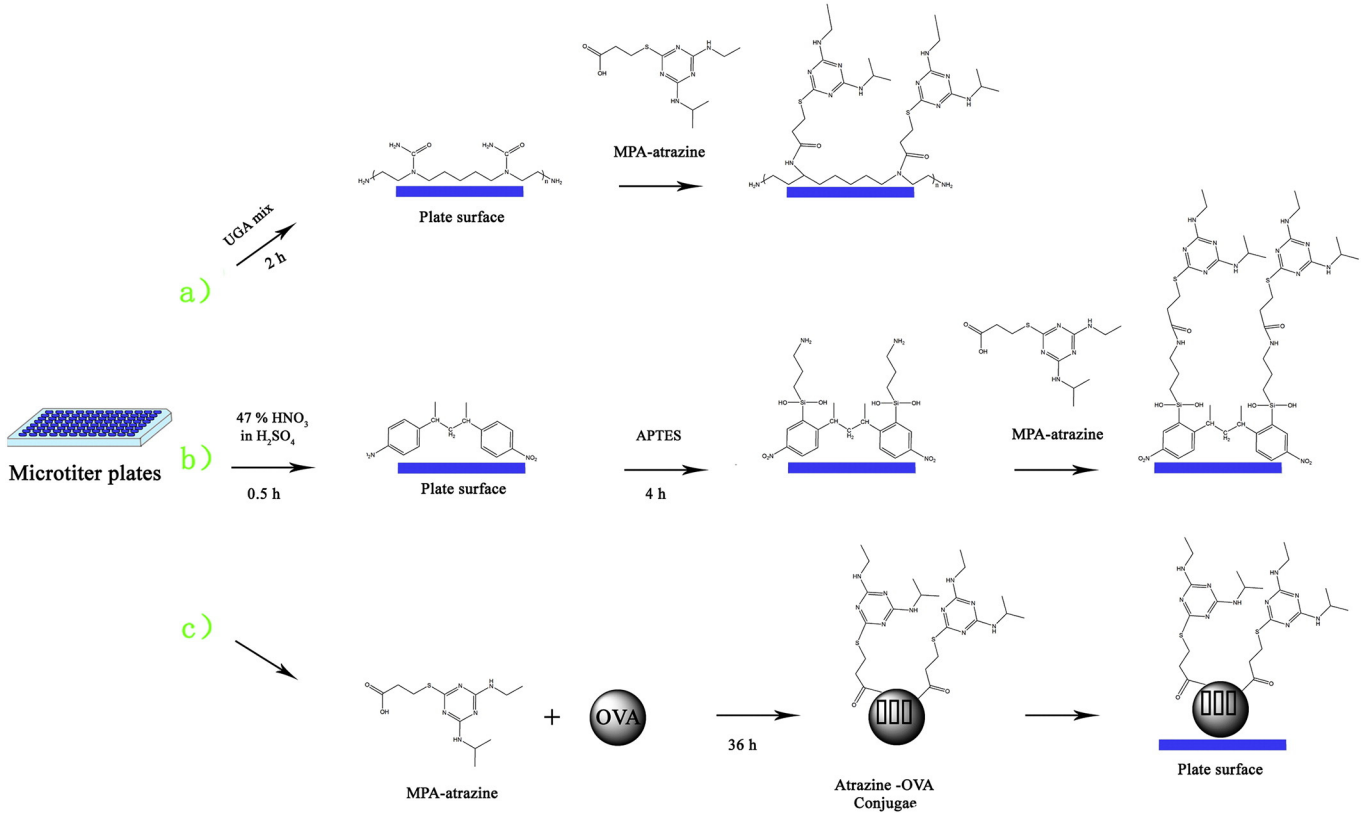

Fig. 1. Reaction mechanism of three different ELISA formats: a) UGA-hapten coated ELISA. The UGA was used to treat the microtiter plate surface for 2 h to introduce amino groups that can covalent link MPA–atrazine; b) HNO_3-H_2SO_4-APTES-hapten coated ELISA. The microtiter plate surface was treated with 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.