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Hapten-Grafted Programmed Probe as Corecognition Element for Competitive Immunosensor to Detect Acetamiprid Residue in Agricultural Products

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ABSTRACT

We have developed an effective competitive electrochemical immunosensor assay based on hapten-grafted programmed probe (HGPP) as a corecognition element for highly sensitive and selective detection of acetamiprid. Starting with the synthesis of hapten, HGPP was prepared using carboxyl group in the hapten and amino group in the 5' end of the programmed probe through covalent conjugation. Acetamiprid present in samples competes with HGPP to bind with capture antibody on the electrodes by specific recognition interaction. Methylene blue probe (MBP) was used as the electrochemical redox probe to capture the hybridized HGPP on the electrodes. The competitive reaction changes in accordance with the quantity of the target acetamiprid in the sample, as the amounts of the hybridized HGPP and the immobilized antibody are constant, i.e., the more acetamiprid samples are added, the less MBP is combined on the electrodes. In the optimal conditions, thus biosensor output a linear relationship from 5 ng L^{-1} to 10^5 ng L^{-1} for acetamiprid assay, with a detecting limit of 3.2 ng L^{-1}. The biosensor was successful in quantifying the amount of acetamiprid in spiked strawberry and cabbage extracts. This competitive immunosensor assay represents a rapid and sensitive technology for acetamiprid assay or other small molecule targets in food.

Keywords: acetamiprid; electrochemical biosensor; competitive immunoassay; hapten; pesticide residues

INTRODUCTION

Food safety issues regarding on pesticides have gained major concerns around the world.[1, 2] Acetamiprid ($C_{10}H_{11}ClN_4$), as one of the most efficient neonicotinoid insecticide, which acts as a stimulant on the insect postsynaptic nicotinic acetylcholine receptors, has been widely used to prevent numerous sucking insects in agricultural products.[3] However, its residue released in soil or accumulated in water and agricultural products might cause potential risk of human health due to its frequent and extensive usage.[4, 5] Therefore, determination of pesticide residues is extremely important for minimizing potential health hazards.

Conventional methods and technologies such as high performance liquid chromatography (HPLC),[6] gas chromatography (GC),[7] mass spectrometry (MS),[8] liquid chromatography-MS (LC-MS),[9, 10] and gas chromatography-mass spectrometry (GC-MS)[11] have found favor in acetamiprid residue analysis. Those methods often provide both quantitative and qualitative dada, but the thermolability and high polarity of acetamiprid make it difficult to analyze using chromatography methods, currently antibody-based immunoassay is preferred for determination in other various matrices through the highly molecular specific recognition interaction of antibody-antigen, as well as integrating highly desirable performance such as specificity, high sensitivity, fast result measurement, and easy-to-operate capabilities.[12] Hence, the most pressing thing is to develop reliable and effective methods that can afford more rapid and high-throughput detection of acetamiprid and other pesticide residues in foods remain.

By the utilization of antigen or antibody as recognition element, various techniques have been developed for toxicological and environmental analyses. Immunoassays offer quantitative analyses based on the highly specific antibody (Ab)-antigen (Ag) interaction.[13-17] Indeed, Ab-Ag

immunoreaction is exploited in various ways including electrochemical,[18] electrochemiluminescence,[19] photo-responsive colorimetry,[20] fluorescence,[21] and surface enhanced Raman scattering (SERS).[22] Among these, electrochemical immunoassays are particularly attractive due to their ease of fabrication and use, high portability and affordability, and low power requirement.[23] Most electrochemical immunoassays to date have focused on the detection of DNA, protein[24-26], microorganisms[27] and other biological entities[28-30]. These targets commonly contain multiple recognition sites and could be detected by conventional sandwich immunoassay, where the immunoreactions happen layer-by-layer with horse reddish peroxidase (HRP)- or glucose oxidase-labeled Ab, the target Ag, and the Ab supported on the substrate.[31, 32] As small molecules are not large enough to combine other antibody molecules too, the detection of drugs or pesticides requires alternate immunoassay strategies.[28, 33-35]

Thus, given the small molar mass of acetamiprid (222.678), we developed a competitive immunoassay for its detection. The acetamiprid-antibody immunocomplex could be indirectly quantitated by monitoring the uncombined sites of the unreacted antibodies. Acetamiprid present in the sample competes with the hapten-grafted programmed probe (HGPP) to bind with capture antibody on the electrodes by specific recognition interaction. Methylene blue probe (MBP) is employed as the electrochemical redox probe based on the hybridization with HGPP on the electrodes. The more acetamiprid samples are added, the less methylene blue is combined on the electrodes. This caused the electric current to decrease in proportion to the quantity of acetamiprid present in the samples. This biosensor detected acetamiprid present in spiked strawberry and cabbage extracts. Our competitive immunosensor has some advantages of rapid detection, easy to be miniaturized, minimal sample consumption, and cost-effective. Therefore, our method might develop

a diverse platform to monitor pesticides and other harmful substances in food with highly sensitive and selective performance.

MATERIALS AND METHODS

Materials and Reagents

HPLC-purified oligonucleotides (the sequences are listed in **Table** S1, Supplementary information) were purchased by Sangon Biotechnology Co. Ltd. (Shanghai, China). Anti-acetamiprid monoclonal Ab was obtained from the Institute of Pesticide and Environmental Toxicology, Zhejiang University (Hangzhou, China). Acetamiprid, chlorpyrifos, methamidophos, omethoate, imidacloprid, 2,4-dichlorophenoxyacetic acid (2,4-D), and carbofuran were purchased from Shanghai Pesticide Research Institute (Shanghai, China). Dihydrolipoic acid (DHLA), 3-mercaptopropionic acid, 1-mercaptohexane (MCH), and potassium hydroxide were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Phosphate-buffered saline (PBS, 20×, pH=7.4), N-hydroxysuccinimide (NHS) 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), and 2-(N-morpholino) ethanesulfonic acid (MES) were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). Others are analytical grade chemicals. Ultrapure water was prepared with a Millipore filtration system.

Apparatus

Electrochemical methods such as differential pulse voltammetry (DPV), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were carried out on a PGSTAT204 electrochemical workstation (The Swiss Wantong Co. Ltd., Holland). A conventional three-electrode system was composed of a bare or functionalized gold (Au) working electrode, a platinum wire counter electrode, and Ag/AgCl reference electrode.

Preparation of Hapten-Grafted Programmed Probe (HGPP)

Hapten was synthesized following a published method[36, 37] as outlined in **Figure** 1A. Then the as prepared hapten was conjugated with programmed probe using the mixed anhydride method.[38] The hapten and trinbutylamine were dissolved in 1 mL dimethyl formamide (DMF). Then 20 μL isobutyl chlorocarbonate was added dropwise under vigorous stirring conditions at room temperature (RT) to react by 2 h. Then the mixture was added dropwise to programmed probe (100 nM) in 2 mL of PBS with stirring and then dialyzed in PBS for 72 hours at 4 °C, and finally reserved at -20 °C.

Preparation of Antibody-Modified Gold Electrode

The preparation of antibody-modified gold (Au) electrode was referred to a previous paper.[39] Clean Au electrode with 2 mm in diameter was subsequently treated for three times by immersing in freshly prepared piranha solution and rinsing with ultrapure water. The pretreated Au electrode was assembled with 1 mM DHLA to obtain the DHLA/Au electrode. Then the mixture of NHS (5 mM) and EDC (2.5 mM) in 0.1 M MES buffer (pH 6.5) was dropped onto the electrode to activate DHLA carboxylic acids. Subsequently, 8 μL of 10 μg mL^{-1} antibody solution was dropped on the surfaces of DHLA/Au electrode and incubated for 2 hours. Unbound antibodies were washed away with 10 mM PBS. Then 8 μL of 2 mM MCH was deposited onto this modified surface for blocking extra sites and reducing non-specific adsorption, and then rinsed by PBS after 30 min to get the MCH/antibody/Au electrode and ready for use.

Acetamiprid Detection via Competitive Immunoassay

The acetamiprid detection was carried out through a typical competitive immunoassay procedure. Different concentrations of acetamiprid solution containing 7.5 μM HGPP was added and incubated with the MCH/antibody/Au electrode at 37 °C for 2h, and then rinsed by PBS to get the

acetamiprid@HGPP/MCH/antibody/Au electrode. Then 8 μL of 7.5 μM MBP solution was drop-coated and incubated with the acetamiprid@HGPP/MCH/antibody/Au electrodes at 37 °C for 2 hours. After rinsed with PBS, the MBP/acetamiprid@HGPP/MCH/antibody/Au electrodes were immersed into 10 mM PBS to perform electrochemical measurements. CV was carried out with parameters of potential (-0.2 V to 0.6 V), step potential (10 mV), and scan rate of (50 mV s^{-1}) in 10 mM PBS containing 5.0 mM $K_3[Fe(CN)_6]$. The DPV measurement was performed within the parameters of potential range (-0.4 V to -0.1 V), step height (4 mV), pulse height (50 mV), and the frequency (15 Hz) in 10 mM PBS.

Preparation of Food Samples

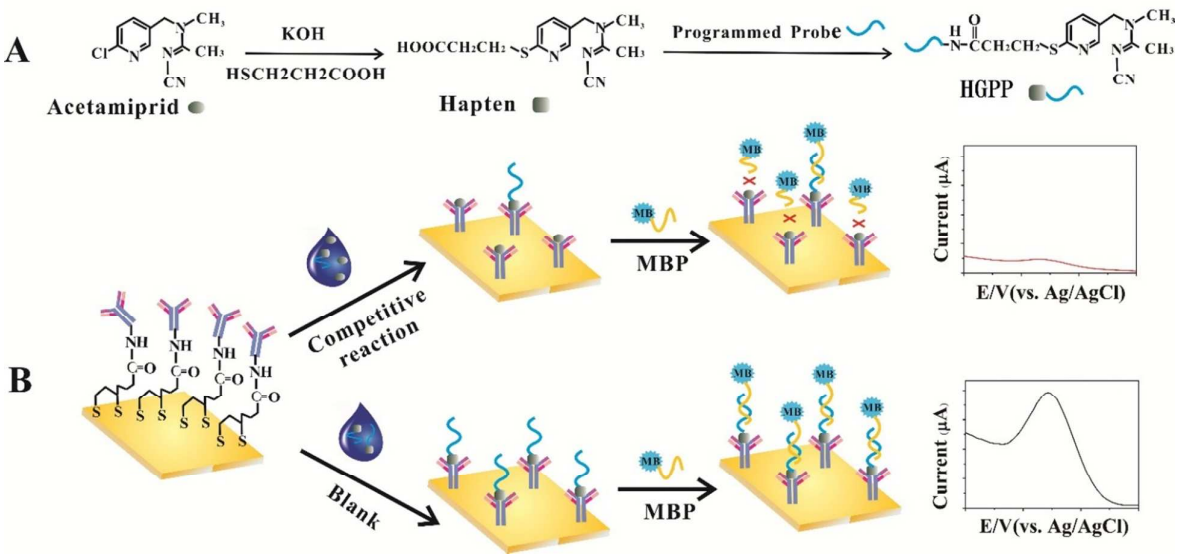
Strawberry and cabbage are the foods most commonly tainted with acetamiprid, hence their ground samples were used as real matrices for acetamiprid detection using the standard addition method. Samples weighted 25 g are stirred by a blender for 2 min with 50 mL water to get the slurries of strawberry and cabbage. Then collect the filtrate into tubes by filter paper to get the pretreated sample solutions. Finally, different concentrations (10^2-10^4 ng L^{-1}) of 100 μL acetamiprid solution were added to 900 μL pretreated sample solutions to obtain test samples of different acetamiprid concentrations of 10-10^3 ng L^{-1}.

RESULTS AND DISCUSSION

Design Principle of the Competitive Immunosensor

The analytical principle of the new simple but effective competitive immunosensor for highly sensitive determination of acetamiprid by combining with HGPP is illustrated in **Scheme 1**. The design of HGPP is the key to preparing a high-quality competitive immunosensor. HGPP synthetic route was illustrated in **Scheme 1**A, and the design method of the hapten molecules for acetamiprid

matches well with the concept of those general strategies.[36, 37] The fundamental design of hapten molecule in acetamiprid by 3-mercaptopropionic acid addresses three major principles, thus to replace a chlorine atom by sulfur atom in each pyridine ring, extend the linker length using two methylene chains from the sulfhydryl compounds, and finally introduce a carboxyl group. Carboxyl group on hapten molecule was performed as the chemical group to covalent conjugate with the free amino group located in the 5' end of programmed probe.[38] As a competitor for acetamiprid, HGPP can adsorb effectively on the surface of antibody by specific recognition interaction. Moreover, the fabrication of the proposed acetamiprid biosensor is illustrated in **Scheme 1**B, such immunosensor is developed based on the capture antibody-immobilized gold electrode via carbodiimide method.[40] The self-assembled monolayer of dithiol-functionalized DHLA enhanced gold surface-binding.[41] Finally, MCH was assembled on electrode surface to hinder extra active sites and reduce non-specific adsorption.



Scheme 1. (A) Preparation of HGPP and (B) competitive immunosensor assay for the detection of acetamiprid.

For testing, samples tainted with acetamiprid and HGPP were then added to induce competitive immunorecognition. Methylene blue probe (MBP) was used as the model of an electrochemical redox probe based on the hybridization with HGPP on the electrodes. The more acetamiprid is present in the test sample, the less MBP combines on the electrodes. This causes the current to decrease, which is used to quantify the amount of acetamiprid present. Therefore, positive sample would prevent signal development whereas nonreactive sample will allow a strong redox signal.

Electrochemical Characterization of the Modified Electrodes

Appropriate modification for sensing interface is important for the biosensor performance. We used EIS to analyze the electrochemical characteristics of the working electrode for each step of modification. The diameter of the semicircle in impedance spectra indicated the electron-transfer resistance (R_{et}),[42] as shown in **Figure 1**A. Before the bare electrode was immobilized with capture antibody, the R_{et} was actually low owing to the direct electron transfer on the sensing interface of bare electrode (curve a). After modifying with capture antibody, an obviously increased R_{et} was achieved, indicating the electron transfer was hindered by the isolated antibodies (curve b). there was an increase of R_{et} when MCH was assembled to block extra active sites, confirming that MCH has been modified to the electrode surface and prevented the electron transfer (as shown by curve c). Followed by addition of target acetamiprid and HGPP, the R_{et} further increased, indicating acetamiprid and HGPP were captured and further blocked the electron transfer on the surface of the work electrode (curve d). After immobilization of MBP, the R_{et} further increased because the electron transfer was hindered by the ssDNA with negatively charged phosphate skeleton (curve e). Moreover, the characterization of the modified procedures was also carried out by cyclic voltammetry. The redox peak current varied by each step of the immobilization in a trend similar to that observed by

R_{et} (as shown in **Figure 1**B). Hence, both results obtained by EIS and CVs have demonstrated that the electrode modification and the biosensing construction was successfully developed for acetamiprid.

Electrochemical Characterization of the Competitive Immunosensing

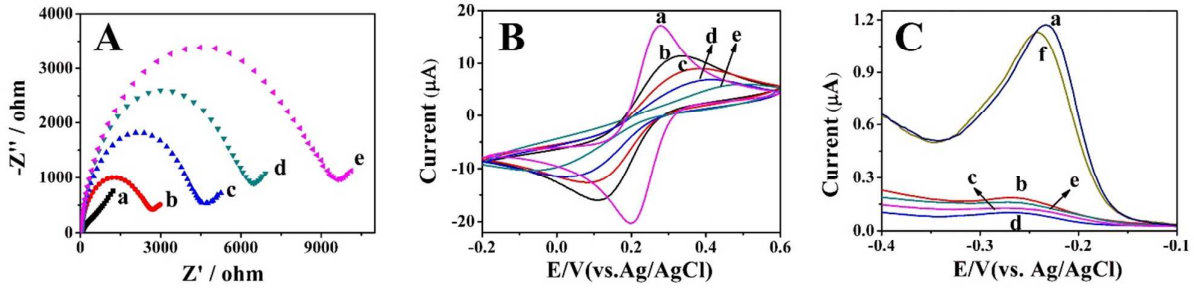


Figure 1. EIS (A) and CVs (B) curves of the electrodes responding to blank control (a), antibody/Au electrode (b), MCH/antibody/Au electrode (c), acetamiprid@HGPP/MCH/antibody/Au electrode (d), MBP/acetamiprid@HGPP/MCH/antibody/Au electrode (e). The sweeping direction of DPV was range from -0.1 V to -0.4 V. (C) DPV responses of competitive immunosensor via the exposure of the modified electrode to blank sample (a), positive sample (b), 50 μg L^{-1} acetamiprid without antibody (c), 50 μg L^{-1} acetamiprid without HGPP (d), 50 μg L^{-1} acetamiprid without MBP (e), 16 mg L^{-1} chlorpyrifos in place of acetamiprid (f). CV and EIS measurements were carried out in 10 mM PBS with 5.0 mM $K_3[Fe(CN)_6]$, and DPV measurements were carried out in 10 mM PBS.

In order to analyze the feasibility of the competitive immunosensor, the electrochemical characterization of the modified electrodes was measured by DPV method in 10 mM PBS. As shown in **Figure 1**C, an extremely strong electrochemical peak displayed at about -0.25 V for blank, suggesting amounts of MBP molecules have captured to the HGPP on the surface of electrode (curve a). While a negligible electrochemical peak appeared under 50 μg L^{-1} acetamiprid, indicating almost

none of MBP was combined (curve b). Results of further control experiments in the absence of antibody (curve c), HGPP (curve d), and MBP (curve e), show negligible peak currents. These results confirm that the competitive reaction and resulted incorporation with MBP was acetamiprid-targeted dependently, rather than induced by other non-specific interferents. Chlorpyrifos is as a model of one kind of pesticides to invest the specificity of this method. After incubation with non-target chlorpyrifos (16 mg L^{-1}) in place of acetamiprid, a significantly strong peak current is obtained, which is similar to the blank sample, demonstrating the specificity of the method (curve f). Upon the above-mentioned results, it is reasonable to conclude that this proposed strategy could be prospectively employed to develop biosensor for acetamiprid assay.

Optimization of Conditions for Competitive Immunosensing

Some crucial experimental parameters including the concentrations of HGPP and capture antibody were investigated to obtain the optimal analytical performance. Typically, high concentration of HGPP could gain high hybridization efficiency with MBP and more significant signal response. Nevertheless, it often results in lower sensitivity and high background signal, which is not beneficial for the quantification of low-abundence of acetamiprid. The relative change was examined in the DPV peak current value, $(I_0\text{-}I)/I_0$, against HGPP concentration, where I and I_0 are currents with or without acetamiprid, respectively (at DPV peak potential -0.25 V). As shown in **Figure 2**A, the maximum value was obtained to be 7.5 μM for HGPP, as the optimal HGPP concentration.

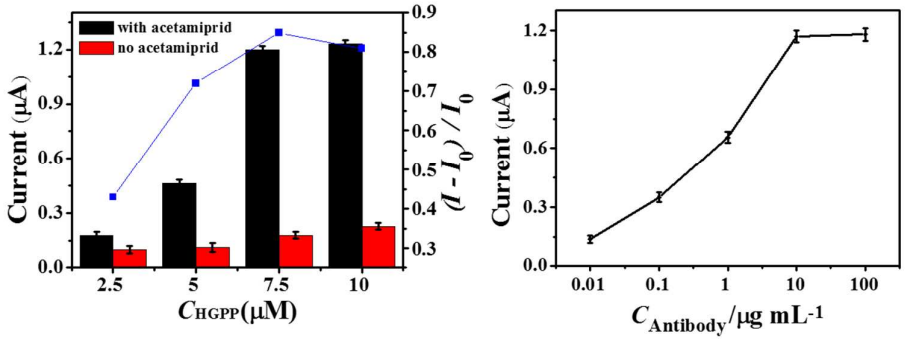


Figure 2. Biosensor current response as functions of (A) HGPP concentration and (B) capture antibody concentration on the electrode.

The surface density of the capture antibody on the electrode is another pivotal factor to improve the sensitivity of the biosensor. **Figure 2**B depicts the effect of the antibody concentration on the DPV current response with 5 ng L^{-1} acetamiprid. As expected, the current signal increased with the antibody concentration, and reached a maximum value at 10 μg mL^{-1} which was adopted as the optimum antibody concentration for all experiments.

Analytical Performance of the Competitive Immunosensor

The constructed immunosensor was employed for detecting a range of various concentrations of acetamiprid under the optimal experimental conditions. Typical DPV signals from the immunosensor to the different concentration of acetamiprid are depicted in **Figure 3**A. The DPV responses decreased with the increasing concentration of acetamiprid. Moreover, there is a good linear relationship between the peak current intensity and the logarithm value of acetamiprid concentration in the range of 5 ng L^{-1} to 10^5 ng L^{-1} (**Figure 3**B), with LOD of 3.2 ng L^{-1} calculated using the equation LOD = $3\sigma/S$, where S is the slope of the calibration curve and σ is the standard deviation of the response at the lowest concentrations. This LOD was much lower than the maximum residue limits (MRL) in GB 2763-2016 China National food safety standard—Maximum residue limit for

pesticides in food (1 mg kg^{-1} for vegetables such as cabbage, 2 mg kg^{-1} for most of fruits, as well as 10 mg kg^{-1} for tea). Comparing with the acetamiprid biosensors reported in the references (**Table** S2), the analytical performance of our competitive immunosensor exhibited greater sensitivity and widely analytic detection range, comparing with most of the literature methods (details are given in the Supporting Information). Besides, the biosensor's performance was highly reproducible. The relative standard deviations (RSDs) of peak intensity were 2.87%, 2.93% and 2.41%, respectively, for three replicate measurements of 10, 10^2 and 10^3 ng L^{-1} of acetamiprid with the same modified electrode. Therefore, owing to its low detection limit, our resulted proposed electrochemical biosensor may fulfill the need to monitor low concentration of pesticides such as acetamiprid in foods.

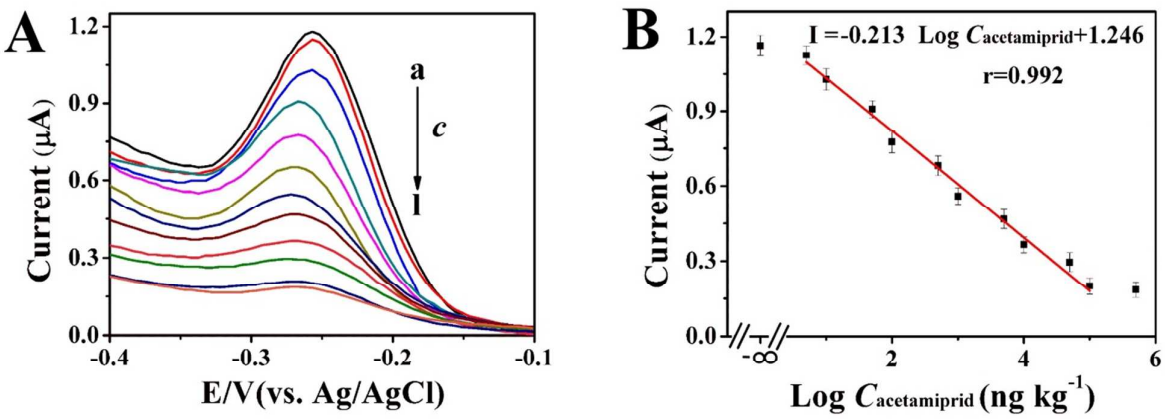


Figure 3. (A) Differential pulse voltammetry curves of the electrochemical biosensor responding to a range of concentrations of acetamiprid (from curve a to l: 0, 5, 10, 50, 10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 5×10^5 ng L^{-1}). (B) Plot of DPV peak current vs the acetamiprid concentrations. The error bars represent standard deviations under three repetitive measurements.

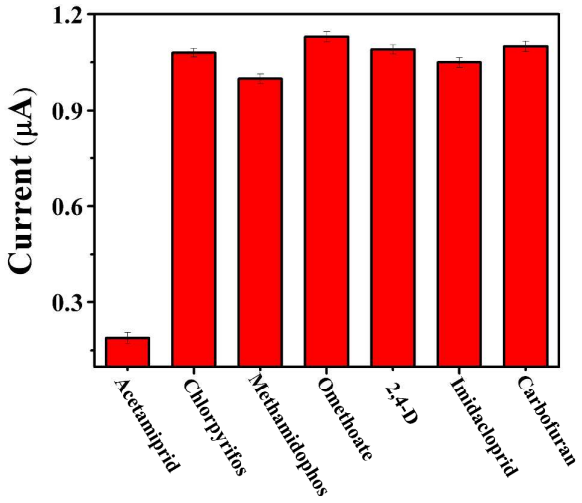


Figure 4. Plot of differential pulse voltammetry peak current procured by different kinds of pesticides. 10^5 ng L^{-1} acetamiprid and 1.6×10^7 ng L^{-1} other pesticides were analyzed. The error bars represent standard deviations under three repetitive measurements.

Moreover, in order to determine the potential application of the proposed biosensor for acetamiprid analysis, six kinds of non-target pesticides were adopted to assess the selectivity of the proposed biosensor. The non-target pesticides, including chlorpyrifos, methamidophos, imidacloprid, omethoate, 2,4-D, and carbofuran were used in over two orders of magnitude higher in concentration compared to that of acetamiprid. According to the results in **Figure 4**, these pesticides does not interfere with the electrochemical performance, indicating superb specificity towards acetamiprid.

Real Sample Analysis

Recovery experiments in real samples were carried out to evaluate the reliability using the standard addition method. Spike the slurries of strawberry and cabbage with different amounts of acetamiprid, respectively. **Table 1** shows the results of recovery for the spiked samples, ranging from 93.7% to 104.3%. It was observed that the recovery for strawberry showed positive impulses above 100%, while the recovery for cabbage showed negative effect below 100%. Maybe the

different result is due to the different acid substrate from different food, which effected the DNA binding or the electrochemical response of MB. These results demonstrated that the proposed sensor could be available to detect pesticides in considerable practical applications.

Table 1. Acetamiprid detection in spiked strawberry and cabbage extracts.

Samples	Spiked amount ($ng\ L^{-1}$)	Sensor measurement ($ng\ L^{-1}$)	Recovery (%)	RSD (%)
Strawberry	10	10.25 ±0.19	102.5	1.89
	100	104.32±3.21	104.3	3.08
	1000	1038.78±30.12	103.9	3.08
Cabbage	10	9.37±0.26	93.7	2.83
	100	98.17±4.6	98.2	4.66
	1000	947.67±18.62	94.8	1.97

CONCLUSION

We have developed a simple but effective competitive immunosensor assay with satisfying performance for acetamiprid assay by using HGPP as co-recognition element. As a competitor for acetamiprid, HGPP can be adsorbed effectively on the surface of antibody by specific recognition interaction. The more acetamiprid is present, the less methylene blue combines on the electrode, causing a decrease in current, which is indicated as the quantity of acetamiprid in the sample. The results reveal that the biosensor response for acetamiprid is approximately linear from 5 ng L^{-1} to 10^5 ng L^{-1} with the LOD of 3.2 ng L^{-1}. Moreover, for most cases of those conventional electrochemical competitive-type immunoassays, the hapten-grafted probes were usually modified with protein like enzyme HRP or other labels. In this work, the HGPP is linked with MB-labelled DNA has achieved a successful detection performance. There are some obvious advantages in this DNA grafted probes strategy. As DNA technology makes it easier for combining small molecules to develop DNA-based

electrochemical biosensor, DNA can be modified with many kinds of electrochemical active substances to release responses directly, thus it does not need to add enzyme reaction substrates and makes the detection process with less steps. While regarding the disadvantages of this HGPP method, it also gives the "Turn-off" signals like most of conventional electrochemical competitive-type immunoassays. We hope to develop better method based on this HGPP competitive strategy in the future.

ACKNOWLEDGEMENTS

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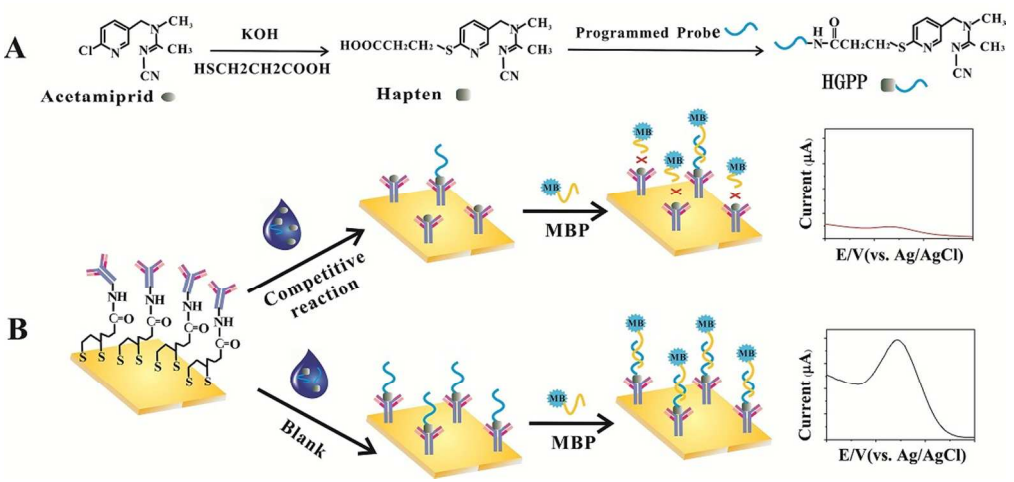

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