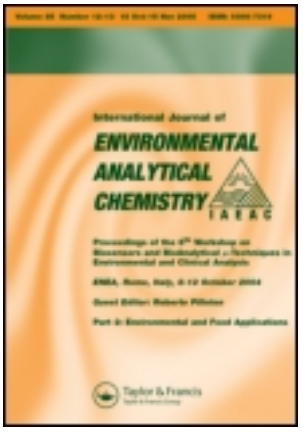

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Sensitive and renewable picloram immunosensor based on paramagnetic immobilisation

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Picloram (4-amino-3,5,6-trichloro-2-pyridincarboxylic acid) is one of the chlorinated pesticides. It is widely used for control of wood plants, wheat, barley and wide range of broadleaf weeds as a plant growth regulator. An immunosensor was developed for detection of picloram concentration in compost extracts and river water. The laccase-picloram was prepared. The magnetic core-shell (Fe_3O_4-SiO_2) nanoparticles were modified with anti-picloram-IgG and attached to the surface of carbon paste electrode (CPE) with the aid of paramagnetism. Following competitive immunoreaction with picloram and the picloram-laccase to form immunocomplex, electrochemical measurement was carried out. After immunoassay, the electrode was immersed in glycin-hydrochloric acid buffer or polished with diamond paper for regeneration. The linear range for picloram detection was 1×10^{-4}–10 $\mu g\,mL^{-1}$ with the correlation coefficient of 0.9936, and the detection limit is 1×10^{-4} $\mu g\,mL^{-1}$. The laccase labelled on the picloram for competitive immunoassay showed good activity, and the current response was strong and stable in electrochemical detection. The current reached 95% of the steady-state current within about 100 s. The proposed immunosensor exhibited good precision, sensitivity, selectivity, reusability, and storage stability.

Keywords: immunosensor; picloram; magnetic nanoparticles; laccase; regeneration

1. Introduction

As they are harmful to human health and the environment, persistent organic pollutants (POPs) have been attracting increasing concern worldwide in recent years. These anthropogenic environmental pollutants are refractory and have long existed in the environment. They threaten the survival of propagation, and some of them may disrupt the natural function of hominine internal secretion. Recently, the negative effects of POPs on human beings have been reported [1–4].

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Most of chlorinated pesticides are classified as POPs due to their high persistence and broad spectrum toxicity [5], such as chlordane, dieldrin, aldrin, etc. They are used extensively in agricultural production and rangeland improvements, transferred into soil substrata, surface and ground waters, accumulated in food chains, and ultimately passed on to human beings [6,7]. Picloram (4-amino-3,5,6-trichloro-2-pyridincarboxylic acid) is one of the chlorinated pesticides that mimic plant auxins or the hormone indoleacetic acid to inhibit the synthesis of proteins [8]. It is widely used for control of wood plants, wheat, barley and a wide range of broadleaf weeds as a plant growth regulator. As indicated in previous toxicological and ecological studies, picloram threatens human health and the environment. High levels of the herbicide can damage the human central nervous system and the reproductive system to a certain extent or cause other health problems [9]. Thus picloram is also suspected to be an endocrine disruptor. Picloram is quite persistent and mobile in soil and water [10,11]. It can remain active for nearly a year in a suitable soil environment, and can easily leach into surface and ground waters. Early in 1998, Close *et al*. found that picloram has strong penetration ability in soil [12]. In view of the hazard and character of picloram, the US Environmental Protection Agency (EPA) has required that the contaminant level for picloram in drinking water should be no more than $0.5\,\mu g\ mL^{-1}$.

Gas/liquid chromatography with electron capture detector and capillary electrophoresis/mass spectrometry are common approaches for picloram detection [8,13,14]; however, large-scale equipment, large sample volumes and extensive extraction or derivatisation steps limit their application in on-site pesticide control. Radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) using the synthetic picloram antibody could also analyse the picloram sample [15,16], but the radiation hazards, large instruments or complicated washing procedure are adverse factors that cannot be ignored in application. In comparison, the electrochemical method possesses many advantages such as high sensitivity, low consumption and easy operation [17–19]. A disposable picloram electrochemical immunosensor was developed by our laboratory. The chitosan/gold nanoparticles composite membrane was used to immobilise picloram for immunoassay with sensitive performance [7].

In recent years, nano-materials have aroused wide concern among researchers of all fields [20–22]. Being able to offer a large specific surface area, retain high bioactivity and keep good electrical conductivity, nano-materials are widely recognised as excellent carriers for biomolecular materials in the analytical chemistry field. Magnetic materials in particular have attracted much attention owing to their inherent magnetic property [23,24]. An interesting immunosensor using $CdFe_2O_4$-SiO_2 nanoparticles to immobilise biomolecules had been presented. The magnetic materials were used to construct the sensing element as a biomolecule carrier and conductor [24]. In addition, laccase is of interest to research scientists. Laccase can directly catalyse the oxidation of a variety of substrate through reduction of oxygen without any other co-substrate [25]. The reaction equations of the redox process on the electrode surface are described as follows:

$$Hydroquinone + O_2 \xrightarrow{laccase} P - Benzoquinone + 2H_2O \quad (1)$$

$$P - Benzquinone + 2e + 2H^+ \longrightarrow Hydroquinone \quad (2)$$

Owing to this property, it would reduce the potential interference of substrate and improve detection efficiency by using this enzyme as an enzyme-label in analysis and detection.

In this study, an electrochemical immunosensor using modified core-shell magnetic nanoparticles to detect picloram was developed. Fe_3O_4 magnetic nanoparticles were synthesised, silylated to form core-shell (Fe_3O_4-SiO_2) structure and functionalised with modified amino groups, and then were cross-linked to anti-picloram-IgG by glutaraldehyde. The immuno-nanoparticles were immobilised on the surface of electrode owing to its paramagnetism. The electrode competitively reacted with picloram and picloram-laccase. In succession, the electrochemical method was used to detect the picloram concentrations with certain concentration of hydroquinone as the response substrate. The competitive immunoassay and electrochemical measurement were optimised to achieve high sensitivity. And the regeneration of the immunosensor was investigated with a combination of immersing regeneration and polishing regeneration to ensure high reusability and facilitate practical application.

2. Experimental

2.1 *Reagents and apparatus*

The analytical standards of picloram, quinclorac, triclopyr and lontrel were purchased from J&K Chemical (Beijing, China). N-hydroxytryptamine (NHS) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC) came from Sigma-Aldrich (St. Louis, MO, USA). N,N-dimethylformamide (DMF), Freund's complete adjuvant (CFA), Freund's incomplete adjuvant (IFA), Bovine serum albumin (BSA) and Horseradish peroxidase-labelled goat antirabbit immunoglobulin G (HRP-G anti-RIgG) were provided by Dingguo Biotechnology Co. (Beijing, China). Glycin was from Biobasic (Markham, Ontario, Canada). Dialysis membrane (15000 MW cutoff) was from Pierce Biotechnology (Rockford, IL). Laccase (EC 1.10.3.2, 23.3 U mg^{-1}) was from Fluka (Buchs, Switzerland). Tetraethoxysilane (TEOS), 3-aminopropyltriethoxysilane (APTES), polyethylene glycol (PEG) and all other chemicals were of analytical grade, and all solutions were prepared in deionised water of 18 MΩ purified using a Milli-Q purification system. The supporting electrolyte was phosphate buffer solution (PBS) prepared with 67 mM KH_2PO_4 and 67 mM Na_2HPO_4.

Electrochemical measurements were carried out on CHI660B electrochemistry system (Chenhua Instrument, Shanghai, China). The three-electrode system used in this work consists of a carbon paste electrode (diameter of 8 mm) as working electrode, a saturated calomel electrode (SCE) as reference electrode and a Pt foil auxiliary electrode. Scanning electron micrographs (SEM) of the CPE surface morphology were obtained with a JSM-6700F field emission scanning electron microscope (JEOL, Japan). A microplate reader (Tecan Sunrise, Switzerland) and 96-well plates (Costar, USA) were used to read the absorbance in the ELISA. A 4K15 Sigma centrifuge (Sigma, Deisenhofen, Germany) and a vacuum freezing dryer were used in the assay. A Model CS501-SP thermostat (Huida Instrument, Chongqing, China) was used to control the temperature. All work was performed at room temperature (25°C) unless otherwise mentioned.

2.2 *Preparation of immunogen, immunisation and purification*

Referring to Fleeker's research [26], picloram was conjugated to BSA. The obtained picloram-BSA was multipoint injected subcutaneously into male white rabbits five times after emulsification. Approximately two months from the initial immunisation, the titer of blood sample reached 1:32, and the rabbit blood was collected. The blood was centrifugated for 15 min at 5000 rpm at 4°C to get the antiserum. Next the antiserum was added in saturated $(NH_4)_2SO_4$ solution to purify by virtue of protein salting-out. The resulting precipitate was dissolved in physiological saline solution and dialysed 48 h in 0.1 M PBS (pH 7.4). The concentration of the stock solution of anti-picloram-IgG was estimated to be 9.3 mg mL^{-1} by measuring the absorbance at 280 nm. The purified immunoglobulin (IgG) was stored at −20°C with the addition of sodium azide (0.1% final concentration).

To evaluate the antiserum reactivity to picloram, each antiserum was titrated against the antigen by immunodiffusion, and indirect ELISA format was carried out with diluted HRP-G anti-RIgG using o-phenylenediamine (OPD) and H_2O_2 as substrates.

2.3 *Preparation of magnetic immuno-nanoparticles*

First, Fe_3O_4 gelatinous precipitate was prepared under nitrogen protection. Next, it was successively added in PEG, water, ammonia solution (28 wt.%) and TEOS under continuous stirring for 24 h, the silica was coated on the magnetic Fe_3O_4 core. After lyophilisation, the core-shell magnetic nanoparticles were suspended in methanol and sonicated for 3 min. APTES was added into the suspension and agitated for 12 h. The precipitates were washed with methanol and water in an ultrasonic bath, and then reacted with 2.5% glutaraldehyde for 3 h [23]. The mixture was centrifugated, and the precipitates were washed with PBS several times and suspended in 5 mL PBS, and then 10 mg anti-picloram-IgG was added in under gentle agitating at 4°C for 12 h. Excess IgG was removed by washing with PBS, then vacuum filtered to obtain the magnetic immuno-nanoparticles and stored in a 4°C refrigerator.

2.4 *Preparation of picloram-laccase and detection of enzyme activity*

The preparation of picloram-laccase is similar to that of picloram-BSA; refer to Supplementary data for details.

The laccase assay was conducted using ABTS as substrate in 0.1 M sodium acetate buffer (pH 5.0). The 2 mL of buffer and 750 μL of 20 mM ABTS and 250 μL of laccase/picloram-laccase solution were taken; the optical density of the oxidation product was read at 420 nm [27].

2.5 *Preparation of the immunoelectrode*

A solid carbon paste electrode was prepared according to the procedure reported elsewhere [28]. First, a kryptol and a caky magnet were put into the polytetrafluoroethylene (PTFE) tube. Next, the carbon paste was stuffed into the PTFE tube to immobilise the magnet. The tube was left to harden for a day. The CPE was polished thoroughly with 0.5-μm diamond paper, sonicated in 1:1 (v/v) nitric acid, acetone and water successively, and rinsed with water before use.

The magnetic immuno-nanoparticles were suspended in 1 mL PBS (pH 7.4), then 40 μL was dripped onto the surface of CPE, dried for 1 h, and the surface was washed with PBS to remove the excessive immuno-nanoparticles. The immuno-nanoparticles were firmly attached to the electrode surface with the help of magnetic force. When not in use, the CPE was stored in a moist state at 4°C.

2.6 *Immunosensor assay procedure*

The strategy of competitive immunoreaction was carried out. Before detection, the CPE was blocked for 30 min in 1 mL 0.1 M PBS (pH 7.4) containing 1% (w/v) BSA, and then immersed in 1 mL block solution containing different concentrations of picloram-laccase and picloram to competitively react for a certain time at 37°C.

The electrolyte was PBS containing certain concentrations of hydroquinone. The cyclic voltammetry was performed between –0.4 and +0.4 V vs SCE at 100 mV/s, and the chronoamperometry was carried out to determine the catalytic activity of the laccase with the reduction current recorded as I_x. When no picloram existed in the competitive immunoreaction, the reduction current was recorded as I_0. The decreased percentage (DP%) of current is given by

$$\mathrm{DP}\ \% = \frac{I_0 - I_x}{I_0} \times 100\% \tag{3}$$

2.7 *Treatment of samples*

The detection performance of the immunosensor could be reflected by using real samples, so compost extract and river water samples were used.

Aerobic compost consisting of soil, straw, canteen residual and bran was built. The carbon nitrogen ratio of the compost was 30:1 and the water content was 65%. A 10 g compost sample and 200 mL water were added into a flask. The suspension was agitated on a mechanical vibrator at 200 rpm for 2 h. The supernatant was centrifuged at 10,000 rpm for 5 min, and then filtered to get the filtrate as the compost extract.

The river water samples were from Xiangjiang River (Changsha, China). In order to get representative samples, the water samples were distributed 10 km along the river which flowed through an urban living area and an agricultural area, and respectively collected in the range of 50 cm under water surface and 50 cm above riverbed within a distance of 1/3 width of the river from the bank. The collected water samples were mixed, filtrated, and then centrifuged for 5 min at 10,000 rpm. The supernatant was used for analysis.

The above two samples were spiked with a certain concentration of picloram which was dissolved in 3% MeOH-PBS (pH 7.4), and then was submitted to analysis.

3. Results and discussion

3.1 *Characterisation of conjugate*

Since amino and carboxyl group coexist in picloram molecule, carbodiimide method was the first choice to prepare complete picloram antigen. To characterise the synthetic

antigen, UV absorption spectrum and infrared spectrum were used. The details are given in the Supplementary data.

A proper ratio of the hapten conjugated to the carrier can give good immunisation results. When the conjugated ratio was low, the specificity of antibody could not be ensured; on the contrary, over-high ratio could change the structure of the protein greatly and reduce the immunogenic activity of the antigen. As for BSA, the molar ratio of hapten to the carrier should be about 20:1. Having a strong absorbance at 277.5 nm, UV absorption spectrum determination of the conjugated compound indicated that the ratio of picloram conjugated to BSA in immunisation was 19.88:1. The conjugated ratio (n) was given by

$$n = \frac{\left(A_{277.5(C)} - A_{277.5(B)}\right)M_B}{\left(A_{277.5(P)} - A_{277.5(C)}\right)M_P} \tag{4}$$

C, *B*, *P* represent the picloram-BSA, BSA and picloram, respectively.

3.2 *Characterisation of antiserum*

The titer of the antiserum increased with the immunisation schedule and reached 1 : 32 after about two months from the first injection. Immunodiffusion test was used to analyse the titer of antiserum. Antibody was diffused towards antigen in the gel matrix and formed a visible precipitated line at proper concentration. No precipitin line was observed when the antibody met with non-affinity antigen. In order to further evaluate the reactivity of the antiserum to picloram, indirect ELISA format was carried out. First, checkerboard assay was used to find the optimal concentrations of HRP-G anti-RIgG and anti-picloram-IgG, then the conventional ELISA procedure was applied. A favourable titer of 1: 4.9×10^5 was obtained which displayed a high level of affinity for picloram.

3.3 *Morphology of electrode surface*

Figure 1 showed the morphologies of electrode surface immobilised on different nanoparticles by SEM. The magnetic core-shell (Fe_3O_4-SiO_2) nanoparticles (a) and the immuno-nanopartiles (b) were immobilised on the surface of CPE with the aid of a permanent magnet. The (a) granular nanoparticles were densely distributed on electrode surface and offered a great specific surface area. Compared with (a), the (b) cluster-shape nanopartiles presented the antibody-wrapped core-shell nanoparticles. The cluster structure indicated that abundant antibodies were conjugated on core-shell nanoparticles which retained the antibodies' bioactivity and significantly improved the immunoreaction ability.

3.4 *Optimisation of detection system*

In order to obtain an optimum performance of the immunosensor, the optimal detection condition of the experiment was studied. After the immunoelectrode was incubated in 0.5 mg mL^{-1} picloram-laccase solution, a cyclic voltammetry scanning was performed. As shown in Figure 2, the immunosensor displayed low background current without notable electrochemical response in blank PBS (pH 4.6). When 10 μL of 0.3 M

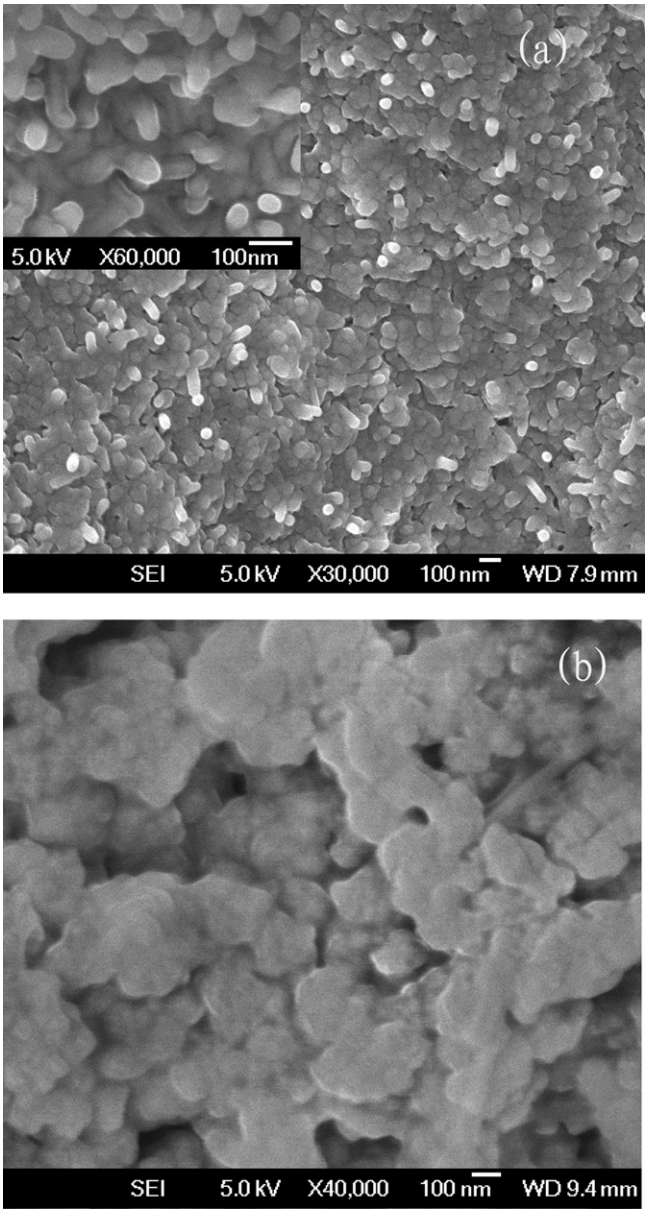

Figure 1. SEM images of (a) magnetic core-shell (Fe_3O_4-SiO_2) nanoparticles and (b) immuno-nanopartiles on surface of electrode.

hydroquinone was added, the reduction current increased prominently in the negative direction and formed a reduction peak, which was attributed to the laccase immobilised on the captured picloram-laccase to oxidise hydroquinone. The mechanism of enzymatic electrocatalysis was reported in our previous study [23]. The reduction peak potential of −0.139 V (vs. SCE) was chosen for chronoamperometry of the immunosensor for picloram.

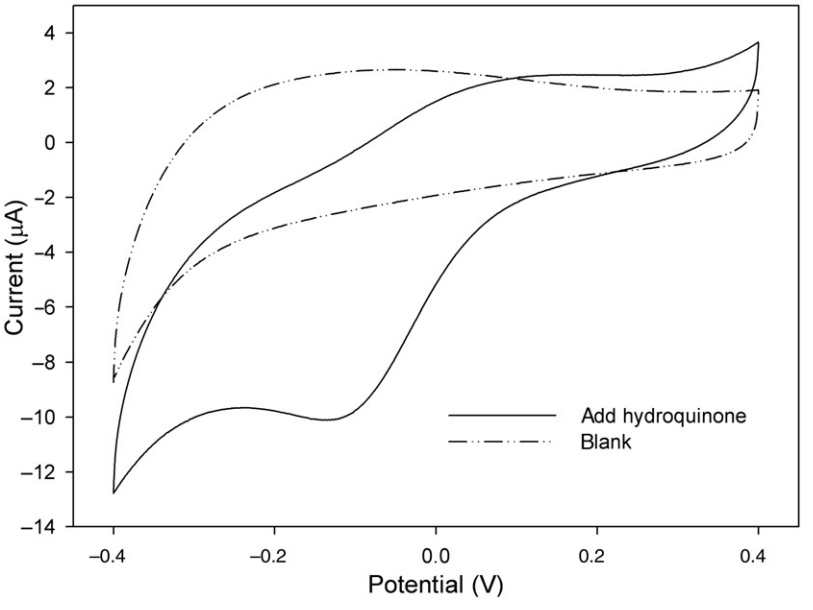

Figure 2. Cyclic voltammograms of the electrode at a scan rate of 100 mV s^{-1} in blank PBS (30 mL, pH 4.6) and 10 μL 0.3 M hydroquinone added.

As signal molecule, the laccase will, if inactive in detection, pose directly impacts on the sensitivity of the biosensor. Most enzymes display high activity only in limited range of pH. Therefore, the influence of pH on the immunosensor response was investigated in the range of 4.0–7.5 by chronoamperometry at the applied potential −0.139 V (vs. SCE). The highest signal-to-background current was detected in the pH range of 4.0–5.0, and in the pH range of 5.0–7.5, the current decreased continuously. When pH was 7.5, the current was close to 0 μA, so the optimum current response was obtained in the pH range 4.0–5.0. In order to achieve the maximum sensitivity, pH 4.6 was chosen in subsequent experiments.

Substrate concentration of the detection solution is another important influence factor. The detection with different hydroquinone concentrations (10–50 μM) was operated in PBS (pH 4.6) at the applied potential −0.139 V (vs. SCE). The current response increased with the increasing hydroquinone concentration and increased very slowly when the hydroquinone concentration reached 50 μM. Therefore, 50 μM hydroquinone was used in this experiment.

3.5 *Optimisation of immunoassay*

The picloram-laccase concentration used in the competitive immunoassay was optimised. The CPE was incubated in a series of concentrations of picloram-laccase solution for 40 min at 37°C. The current response increased with the increasing labeled hapten concentration and reached maximum at 1 mg mL^{-1}. In this series of assays, laccase labelled on the picloram showed good activity, and the current response was strong and stable. Therefore, 1 mg mL^{-1} picloram-laccase was chosen for further study.

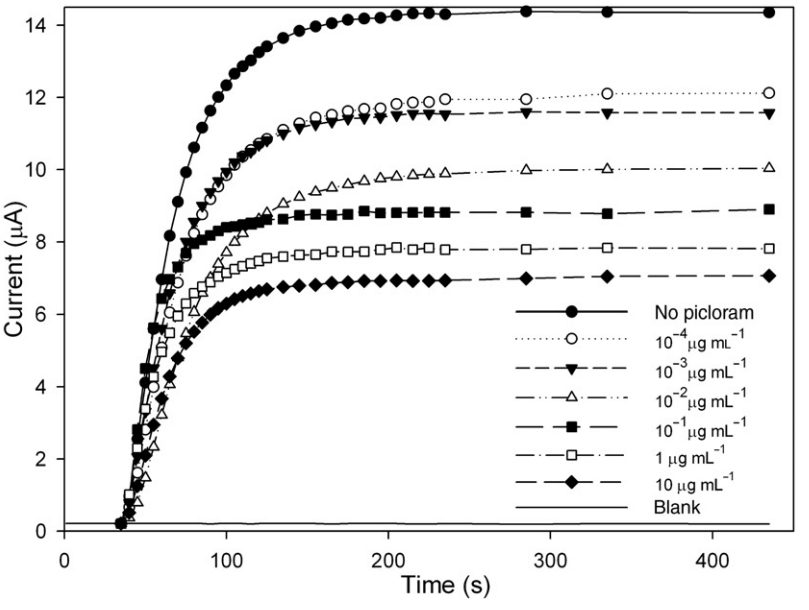

Figure 3. Amperometric curves of different picloram samples in PBS (pH 4.6) containing 50 μM hydroquinone at an applied potential of −0.139 V vs. SCE.

Another important experimental factor to immunoassay is incubation time, which is sure of full immunoreaction. The incubation time range from 10 to 60 min was investigated. During the incubating process, the pH value was kept at 7.4, and incubating temperature was 37°C, which simulated the physiological environment of organism to provide a natural condition to ensure the physiological function of IgG. The response current increased with the increasing incubation time, and reached a platform at 40 min. Therefore, 40 min was adopted as the incubation time for competitive immunoreaction in the following immunoassays.

3.6 *Characterisation of immunosensor response*

Under the optimal immunoreaction and detection conditions, the target picloram and picloram-laccase would compete with the IgG immobilised on the electrode surface to form immunocomplex. Figure 3 showed the current-time curves of the immunosensor under different picloram concentrations. The current responses were rapid, clearly detectable and recorded when the steady-state was reached with low average background current values. The current reached 95% steady-state within 100 s. The decrease percentage of amperometric current response attributed to the competitive immunoreaction increased with an increasing picloram concentration (Figure 4). The average decrease percentage DP(%) was linearly related to the natural logarithm of picloram concentration^{x} ($\mu g\,mL^{-1}$), ranging from 1×10^{-4} to $10\,\mu g\,mL^{-1}$ with the following regression equation:

$$DP\% = (3.6966 \pm 0.1399)x + (44.8445 \pm 0.683) \quad (5)$$

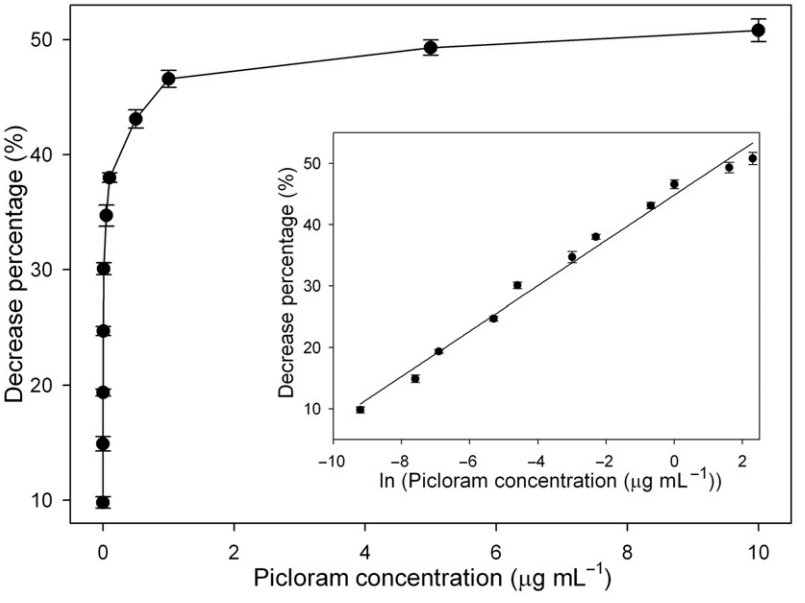

Figure 4. Calibration curve of immunosensor for picloram determination under the optimal experimental conditions. The vertical bars designate the standard deviation for the mean of three replicate tests. (Inset) Linear regression of decrease percentage vs. natural logarithm of picloram concentration.

The correlation coefficient was 0.9936. The detection limit was $1 \times 10^{-4}\,\mu g\,mL^{-1}$ at a signal-to-noise of three (S/N = 3). Calibration was triplicated for corresponding picloram concentration, and the relative standard deviations (RSDs) were 2.31% and 4.57% for five replicates at the picloram concentration of 0.05 and $1\,\mu g\,mL^{-1}$, respectively, which guaranteed the precision and reproducibility of the immunosensor.

3.7 *Detection of picloram in sample*

As herbicide, picloram is widely used around the world. It is quite persistent and mobile in soil and water, and liable to transfer to plants, microbes and the related food chain ecosystem. Therefore, it is possible to exist in environmental pollution related industry, agriculture and horticulture. Compost and river water samples were spiked with picloram. For the detection of real samples, the matrix effect of samples should be circumvented [29]. To reduce the matrix effect and avoid excessive content of picloram in samples, the real samples were diluted tenfold by PBS (pH 7.4) before detection. A small amount of MeOH was used to ensure full dissolution of picloram, and its potential influence could be reduced significantly due to the evaporation of MeOH. The recoveries of picloram from the samples were shown in Table 1. There was little or no matrix effect to compost and the Xiangjiang River water samples. The results indicated the suitability of the immunosensor as a simple, fast and reliable detection method of picloram in the environmental samples.

Table 1. Recovery of picloram from compost extract and river water samples using the immunosensor.

Sample	Concentration added ($\mu g m L^{-1}$)	Concentration found ($\mu g m L^{-1}$)	Recovery (%)	CV (%)
Compost extract	0.032	0.035 ± 0.003	113.6	10.4
	0.29	0.287 ± 0.010	99.0	7.2
	1.22	1.203 ± 0.32	98.6	9.1
Xiangjiang River water	1.0	1.007 ± 0.10	100.7	8.7
	0.065	0.064 ± 0.021	98.5	9.8
	0.025	0.027 ± 0.005	108	11.6

3.8 *Regeneration and stability*

The regeneration of the sensor is an important technical parameter, which reflects whether the sensor possesses reusability. The CPE was regenerated by immersing it in 0.2 M glycin-hydrochloric acid buffer (pH 2.4) for 5 min. Glycine could effectively dissociate the antigen–antibody complex, peeling the captured antigens off the immunoelectrode. In addition, hydrochloric acid would provide an appropriately high ion-strength to facilitate the detachment of antibodies and antigens [30,31]. To verify the regeneration effect, 1 $\mu g m L^{-1}$ picloram was detected by chronoamperometry, followed by regeneration with the buffer. The above steps were repeated four times with the standard deviations of current response not more than 4%. It showed that the regeneration method was effective. After using this method for about 20 times, the effect of the regeneration reduced, so carbon paste was extruded a little and polished with 0.5 μm diamond paper. Then the electrode was reproduced as described in section 2.5 above. The combination of the two methods guaranteed the immunosensor possesses good reusability and convenience for application.

The CPE was stored in a moist state at 4°C when not in use. The stability of the immunosensor was investigated by measuring the amperometric response to 1 $\mu g\ mL^{-1}$ picloram. The response current of the immunosensor showed no apparent change during the period of about 12 days.

3.9 *Interference*

To evaluate the selectivity of the immunosensor, some possible interfering substances with similar structure, such as quinclorac, lontrel, triclopyr and pyridine, were examined under the same condition for the determination of picloram. Compared with the response current change of immunosensor to picloram, the relative response current changes were 1.28% for quinclorac, 1.54% for lontrel, 1.61% for triclopyr and 5.03% for pyridine. It can be inferred that these substances in lower concentrations do not cause interference. So the picloram immunosensor showed good selectivity.

4. Conclusion

An immunosensor was developed for the detection of low levels of picloram in environmental samples based on the anti-picloram-IgG immobilised on the modified

magnetic core-shell nanoparticles surface. The anti-picloram-IgG was covalently immobilised onto the magnetic nanoparticles and then attracted to CPE surface by dint of paramagnetism. And laccase was conjugated to picloram as marker to complete the competitive immunoassay. The optimal experimental conditions for the operation of the immunosensor were studied. The results showed that the immunosensor exhibited good sensitivity, stability, reusability, selectivity and other obvious advantages for picloram determination. The anti-picloram-IgG used in this study was polyclonal antibody; if it is replaced by monoclonal antibody which possesses stronger immune affinity, better detection results would be obtained. The immunosensor was not only capable of efficiently detecting low levels of picloram in environmental samples, but could be readily extended towards the on-site monitoring for other trace toxic organic pollutants and genes in environmental matrices in further studies.

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