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Electrogenerated Chemiluminescence Immunosensor for *Bacillus thuringiensis* Cry1Ac Based on Fe_3O_4@Au Nanoparticles

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ABSTRACT: A highly sensitive electrochemiluminescence (ECL) immunosensor for Cry1Ac was fabricated. The primary antibody anti-Cry1Ac was immobilized onto core−shell structural Fe_3O_4@Au nanoparticles. The antigen and glucose-oxidase-labeled secondary antibody were then successively combined to form sandwich-type immunocomplexes through a specific interaction. The magnetic particles loaded with sandwich immune complexes were attracted to a magnet-controlled glass carbon electrode (GCE) by an external magnet applied on top of the GCE. ECL was generated by the reaction between luminol and hydrogen peroxide derived from the enzymatic reaction in the presence of glucose. The sensors exhibited high sensitivity and a wide linear range for *Bacillus thuringiensis* Cry1Ac detection from 0 to 6 ng/mL, as well as a detection limit of 0.25 pg/mL (S/N = 3). The sensor is one of the most sensitive sensors for Cry1Ac, which can be easily renewed and conveniently used.

KEYWORDS: *Cry1Ac proteins, immunosensor, electrochemiluminescence, magnetic nanoparticles, luminol*

INTRODUCTION

Genetic modification (GM) refers to the transfer into and expression of genes in receptor organisms or cells.[1] Numerous edible genetically engineered plants are commercially used. Since the establishment of the transgenic technique, its food, environmental, ecological, and social risks have been debatable. These safety issues have important implications for the detection of GM ingredients in plant products.

Bacillus thuringiensis (Bt) is an aerobic, spore-forming soil bacterium that produces highly specific insecticidal proteins named δ-endotoxins.[2,3] At the end of sporulation, both spores and crystals are liberated.[4] The possible crop yield losses because of pests account for 13% of the total harvests worldwide. Currently, chemical control is the most commonly used method against pests. However, this method leaves large agricultural chemical residues that may harm livestock.[5] Pests also easily acquire resistance to existing chemicals. Therefore, finding a new biological pest control is urgently needed. Cry1Ac proteins are considered to be environmentally friendly[6,7] and have been used for pest control for many years in forestry management, agriculture, as well as vector-borne disease control. Cry toxins are the major insecticidal proteins in Bt.[8] Bt genes that code insecticidal toxins are the primary transgenes in current transgenic crops.[9−11] Cry1Ac protein toxin was extracted from Bt Kurstaki HD-73.[12] In the perspective of food safety, a sensitive analytical method for Cry1Ac detection in transgenic plants is important to establish. At present, many methods[13] are available for transgenic Bt toxic protein Cry1Ac detection, such as immunoassay polymerase chain reaction (PCR),[14,15] electrochemical methods,[16] enzyme-linked immunosorbent assay (ELISA),[12,17−20] and capillary electrokinetic chromatography.[21] However, the detection performance of these methods for transgenic production is time-consuming,[22] laborious, and costly. Electrochemiluminescence (ECL) immunoassay, which combines the highly sensitive ECL and immunoassay techniques,[23] has high sensitivity, selectivity, and specificity. However, an electrogenerated chemiluminescence immunosensor for transgenic plant proteins, particularly for Bt Cry1Ac, has not yet been reported.

Because of their high specific surface area, high surface reactivity, large quantity of surface active centers, and high catalytic efficiency, nanoparticles (NPs) can increase the adsorption and stability of a biologically active substance.[24] Magnetic NPs are excellent carriers of biologically active substances because of their special physicochemical properties.[25−27] The surface functionalization and modification of magnetic NPs to introduce additional functionality of enzymes, antibodies, and electroactive reagents is gaining increasing attention.[28−30] The most widely studied NPs is magnetite Fe_3O_4,[31,32] which offers additional properties, such as superparamagnetism, low toxicity, and simple preparation. Fe_3O_4 NPs[33] are often modified by Au NPs with a core−shell structure that can not only prevent Fe_3O_4 NP aggregation but also increase stability and compatibility. The Au NPs can provide a platform for surface modification and function[34] to prepare the core−shell structure of Fe_3O_4@Au NPs.

An ECL biosensor based on Fe_3O_4 magnetic particles has been reported with high sensitivity and the easy renewal of the electrode surface by our group.[35] In this paper, we developed a novel ECL immunosensor based on Fe_3O_4@Au paramagnetic NPs and glucose oxidase (GOD)-labeled antigen. Fe_3O_4@Au was functionalized by primary antibody, and GOD-labeled secondary antibody was used to form a sandwich construction. Hydrogen peroxide (H_2O_2) was produced by an enzymatic reaction catalyzed by GOD.[36] ECL of luminol was initiated by applying an appropriate positive potential to the working electrode in the presence of H_2O_2.[37−39] The adsorption quantity of Cry1Ac significantly increased because of the

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Figure 1. Schematic of the ECL biosensor.

large specific surface area and good biocompatibility. The enzyme-labeled antibody technique was introduced, and the signals were amplified. The results showed high performance (high sensitivity, renewability, and versatility) for the detection of the Cry1Ac protein.

■ MATERIALS AND METHODS

Apparatuses and Reagents. Cyclic voltammetric experiments and ECL measurements were carried out on a model MPI-E ECL analyzer (Xi'an Remex Instrument Co., Ltd., China) with a three-electrode system that consisted of a platinum wire as an auxiliary electrode, an Ag/AgCl electrode as the reference electrode, and a Cry1Ac immunosensor as the working electrode. A pHS-2C model pH meter (Shanghai Leici Instruments, China) and a DK-8B electro-thermal constant temperature incubator (Shanghai Jinghong Instruments, China) were also used.

Transgenic Bt toxic protein Cry1Ac, primary antibody, and GOD-labeled secondary antibody (0.6 μg/mL) were obtained from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (Beijing, China). GOD (120 units/mg, from *Aspergillus niger*) was purchased from Sigma. Gold chloride ($HAuCl_4$) was purchased from Sinophram Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) and glutaraldehyde (25%) were obtained from Shanghai Biochemical Co., Ltd. (Shanghai, China). $FeCl_3 \cdot 6H_2O$ was obtained from Xilong Chemical Works (Guangdong, China). $FeSO_4 \cdot 7H_2O$ was produced by Heshan Chemical Works (Guangdong, China). Thiourea was from Tongguang Fine Chemicals Company (Beijing, China).

A 0.01 mol/L luminol stock solution was prepared by dissolving 0.0886 g of luminol (>98%, Fluka) in 0.1 mol/L sodium hydroxide buffer. Phosphate-buffered solution (PBS, pH 7.4) was prepared using 0.1 mol/L Na_2HPO_4, 0.1 mol/L NaH_2PO_4, and 0.1 mol/L NaCl. The washing buffer solution consisted of PBS containing 0.1 mol/L NaCl and 0.05% (v/v) Tween 20 (PBST). Approximately 0.05 mol/L tris(hydroxymethyl)aminomethane and 0.1 mol/L HCl were used to prepare the Tris-HCl buffer solution.

All other reagents were analytical-reagent-grade. All solutions were prepared with doubly distilled water (18.2 MΩ cm^{-1}).

Synthesis of Fe_3O_4@Au Composite NPs. Fe_3O_4@Au NPs were prepared by a two-step method according to the procedures reported by our group.[40] The first step was the synthesis of Fe_3O_4 seeds prepared by the chemical co-precipitation of Fe^{II} and Fe^{III} ions (2:1 molar ratio) in alkaline medium. NaOH solution (2 mol/L) was added to ferric and ferrous chloride under vigorous agitation at 50 °C. During the reaction process, the pH value was maintained at about 10. The solution was then heated at 80 °C for 1 h under a N_2 atmosphere. Finally, the resulting precipitate was separated by magnetic decantation and washed with doubly deionized water.

The second step involved the synthesis of Fe_3O_4@Au NPs prepared by the reduction of Au^{3+} using Fe_3O_4 particles as seeds. Under constant stirring, 100 mL of sodium citrate (2.29 g/mL) was prepared and heated at 90 °C. Then, 40 mg of Fe_3O_4 NPs was immediately added to the solution. About 5 mL of $HAuCl_4$ solution (0.01 mol/L) was added and heated for 15 min before cooling to room temperature with vigorous stirring for 15−20 min. The obtained colloidal solution was isolated in a magnetic field. The magnetically separated Fe_3O_4@Au NPs were rinsed and suspended in 20 mL of doubly deionized water.

Anti-Cry1Ac Conjugated to Fe_3O_4@Au NPs. About 2 mL of Fe_3O_4@Au particles was added to 10 mL of 10 mmol/L thiourea under constant stirring and reacted for 120 min. After magnetic separation, 2 mL of glutaraldehyde was added and reacted for 1 h. The particles were thoroughly cleaned using PBS buffer. Subsequently, 50 μL of Cry1Ac (0.1 mg/mL) solution was added and diluted to 2 mL and then reacted for 120 min under constant stirring at 4 °C. Finally, anti-Cry1Ac/Fe_3O_4@Au was obtained and refrigerated until use.

Preparation of Immunosensors. The modified electrode was prepared according to a published procedure.[35] The core of the electrode was attracted by a magnet. The fabrication processes of the ECL immunosensor are shown in Figure 1. During the procedure, 20 μL of anti-Cry1Ac/Fe_3O_4@Au suspension was added dropwise onto the electrode surface and allowed to disperse. The electrode was subsequently immersed in 1% BSA solution to seal the non-specific sites on the particle surface and then rinsed with PBST. The electrode was then incubated with Cry1Ac (<6 ng/mL) for 30 min. Finally, a sandwich immunoconstruction was formed by incubation in 21 ng/mL GOD-labeled anti-Cry1Ac for 30 min. The excess antibody was washed with PBST. The immunosensor was easily renewed by removing the biomagnetic particles without the magnet and rinsing with 0.5 mol/L HCl.

ECL Measurement. The ECL test was conducted in 10 mL of 0.05 mol/L Tris-HCl buffer (pH 8.5) containing 0.6 mmol/L luminol and 1 mmol/L glucose at room temperature using a model MPI-E ECL analyzer. The ECL measurement was performed from −0.3 to 0.6 V at the scan rate of 100 mV/s. The photomultiplier tube (PMT) voltage was set at 800 V. The ECL signal−time curve was obtained under continuous potential scanning for five cycles at 4× magnification to record the ECL signals related to the Cry1Ac concentrations.

Sample Treatment. Approximately 100 g of fresh leaves of genetically modified soybean (HD-73) were collected. For the composite samples of the transgenic and non-transgenic mixture, 50 mg of fresh leaves of the genetically modified soybean (HD-73) were mixed in 100 g of non-transgenic soybean leaves. The leaf samples were added to 50 mL of buffer A [containing 10 mmol/L Tris-HCl (pH 7.5), 2 mmol/L mercaptoethanol, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (pH 8.0)] and crushed. The sample was then degreased by diethyl ether; buffer A was added; and the supernatant was collected after stirring for 120 min and centrifugation (4 °C, 10 000 revolutions/min) for 15 min. The protein in the supernatant solution was precipitated by 50% $(NH_4)_2SO_4$ and then allowed to settle for 30 min. After centrifugation (4 °C, 10 000 revolutions/min), the supernatant was discarded. The resulting precipitate was dissolved and dialyzed by 10 times the volume of buffer A overnight at 4 °C. Finally, the sample was isolated through a Sephadex G-25 chromatography column and DEAE-Toyopearl.

■ RESULTS AND DISCUSSION

Characterization of Fe_3O_4@Au. Scanning electron microscopy (SEM) in the secondary electron mode of Fe_3O_4@Au

was carried out using a Hitachi S-4800 field emission SEM (FESEM). The SEM image is shown in Figure 2a. The particle

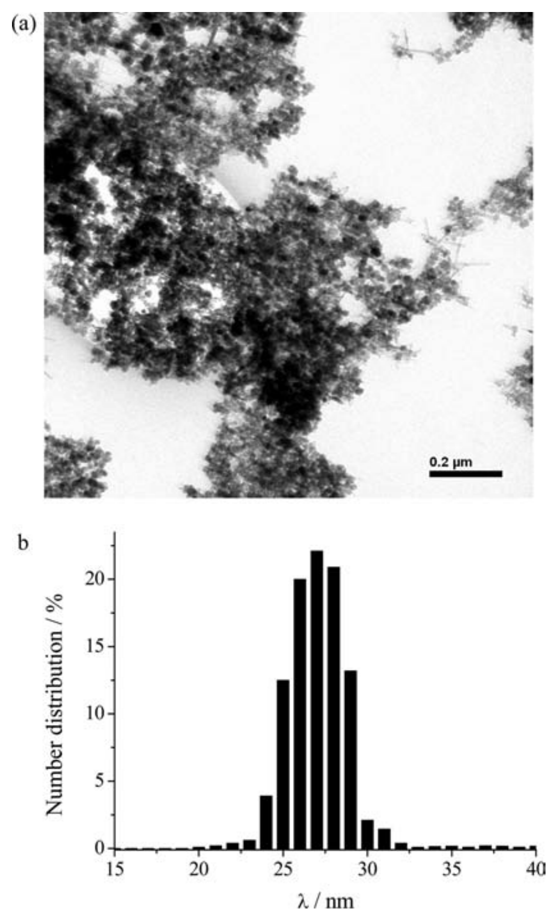

Figure 2. (a) SEM and (b) particle size distribution graph of Fe_3O_4@Au.

size distribution graph of Fe_3O_4@Au was obtained using a Zetasizer Nano Series analyzer (Zetasizer Nano ZS90, Malvern, U.K.), with a 50 mV laser. The graph is shown in Figure 2b. The average diameter of the core−shell of Fe_3O_4@Au was about 25−30 nm.

Cyclic Voltammetry (CV) and ECL Responses. The CV and corresponding ECL intensity curves are shown in Figure 3. High current response (a) and weak ECL intensity (a_0) were observed at the bare electrode. On the anti-Cry1Ac/Fe_3O_4@Au-modified electrode, the current decreased (b) and light emission slightly increased (b_0) in the absence of the Cry1Ac antigen because of the adsorption of GOD-labeled anti-Cry1Ac. When the Cry1Ac antigen was added, the current obviously decreased (c) and the ECL intensity sharply increased (c_0). This finding demonstrated that GOD-labeled anti-Cry1Ac was successfully bound onto the surface of the magnetic NPs attached on the electrode.

Optimization of Cry1Ac Immunoassay. The ECL behavior was investigated in 0.1 mol/L borax buffer solution, 0.05 mol/L Tris-HCl buffer solution, and 0.1 mol/L PBS (pH 8.0).

The results demonstrated that the maximal ECL intensity can be obtained in Tris-HCl buffer solution. The effect of the Tris-HCl buffer pH on the ECL intensity was investigated within the range of 6.5−9.5, and the results are shown in Figure

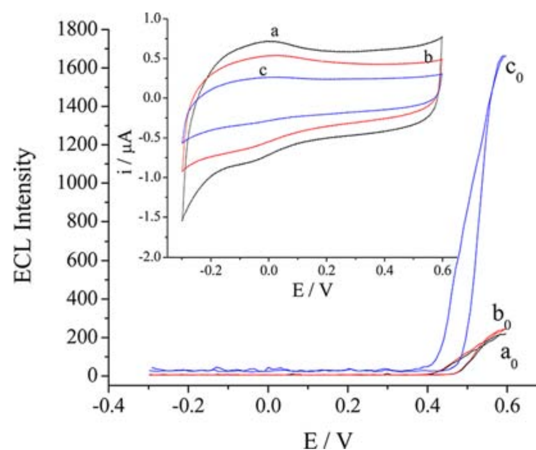

Figure 3. (a_0, b_0, and c_0) ECL curves and (a, b, and c) cyclic voltammograms (inset): (a and a_0) GCE in 1 mmol/L glucose, 0.6 mmol/L luminol, and 0.05 mol/L Tris-HCl buffer solution, (b and b_0) anti-Cry1Ac/Fe_3O_4@Au/GCE with 20 μL of anti-Cry1Ac/Fe_3O_4@Au particles in the solution of a, and (c and c_0) immunosensor in the solution of a + 0.4 ng/mL Cry1Ac.

4a. A higher ECL intensity of luminol can be achieved in alkaline solution. The ECL signal steeply increased with an

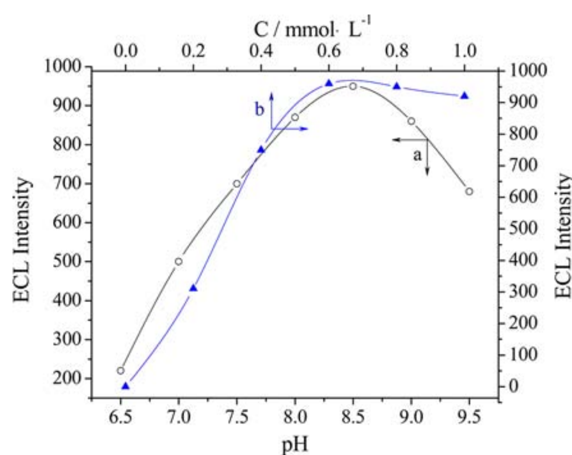

Figure 4. Effect of (a) pH and (b) luminol concentration on the ECL intensity: (a) 0.05 mol/L Tris-HCl buffer containing 0.6 mmol/L luminol and 1 mmol/L glucose, incubation at 35 ± 1.0 °C for 30 min, 0.2 ng/mL Cry1Ac, 25 μL of anti-Cry1Ac/Fe_3O_4@Au, and 21 ng/mL GOD-labeled anti-Cry1Ac and (b) 0.05 mol/L Tris-HCl buffer (pH 8.5) containing and 1 mmol/L glucose, incubation at 35 ± 1.0 °C for 30 min, 0.2 ng/mL Cry1Ac, 25 μL of anti-Cry1Ac/Fe_3O_4@Au, and 21 ng/mL GOD-labeled anti-Cry1Ac.

increased pH, reached the maximum at pH 8.5, and then decreased when the pH was higher than 8.5. Hence, pH 8.5 was selected as the optimal pH.

The effect of the luminol concentration within the range of 0−1.0 mmol/L on the ECL intensity was also investigated. Figure 4b shows that the ECL intensity increased with an increased luminol concentration and tended to be constant beyond 0.6 mmol/L. Hence, the optimal luminol concentration was 0.6 mmol/L.

The effect of the incubation time and temperature on the ECL density was subsequently evaluated, and the results are illustrated in Figure 5. The ECL signal markedly increased with an increased incubation time used in the sandwich-type

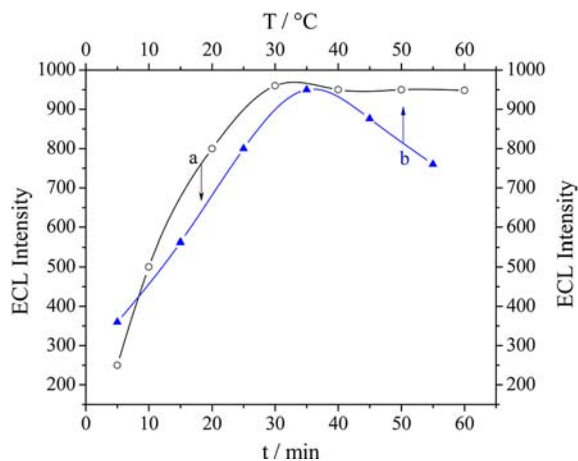

Figure 5. Effect of (a) incubation time and (b) incubation temperature on the ECL intensity: (a) 0.05 mol/L Tris-HCl buffer (pH 8.5) containing 0.6 mmol/L luminol and 1 mmol/L glucose, incubation at 35 ± 1.0 °C, 0.2 ng/mL Cry1Ac, 25 μL of anti-Cry1Ac/Fe_3O_4@Au particles, and 21 ng/mL GOD-labeled anti-Cry1Ac and (b) 0.05 mol/L Tris-HCl buffer (pH 8.5) containing 0.6 mmol/L luminol and 1 mmol/L glucose, incubation for 30 min, 0.2 ng/mL Cry1Ac, 25 μL of anti-Cry1Ac/Fe_3O_4@Au particles, and 21 ng/mL GOD-labeled anti-Cry1Ac.

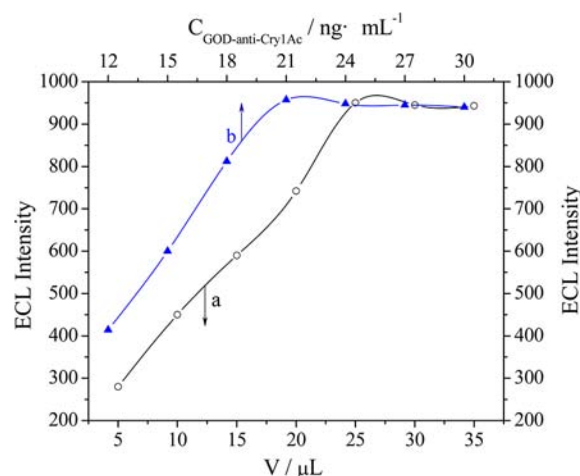

Figure 6. Influence of (a) anti-Cry1Ac/Fe_3O_4@Au particle volume and (b) concentration of GOD-labeled anti-Cry1Ac on the ECL intensity: (a) 0.05 mol/L Tris-HCl buffer (pH 8.5) containing 0.6 mmol/L luminol and 1 mmol/L glucose, incubation at 35 ± 1.0 °C for 30 min, 0.2 ng/mL Cry1Ac, and 21 ng/mL GOD-labeled anti-Cry1Ac and (b) 0.05 mol/L Tris-HCl buffer (pH 8.5) containing 0.6 mmol/L luminol and 1 mmol/L glucose, incubation at 35 ± 1.0 °C for 30 min, 0.2 ng/mL Cry1Ac, and 25 μL of anti-Cry1Ac/Fe_3O_4@Au particles.

immunoassay and then slowly changed after 30 min (Figure 5a). This change meant that the binding of the antigen and antibody was saturated. Therefore, 30 min was selected as the optimal incubation time for the immunoassay.

The ECL intensities were detected from 5 to 55 °C (Figure 5b), and the maximum response was obtained at 35 °C. Considering the reduction of immunosensor life at high temperatures, the incubation temperature was maintained at 35 ± 1.0 °C.

Given that the ECL response can be initiated in the presence of GOD, the anti-Cry1Ac/Fe_3O_4@Au volume effect was assessed from 5 to 35 μL. When the volume of anti-Cry1Ac/Fe_3O_4@Au was larger than 25 μL, ECL ceased to increase (Figure 6a); hence, 25 μL was selected as the optimal volume.

The GOD-labeled anti-Cry1Ac concentration was examined from 12 to 30 ng/mL, and the results are shown in Figure 6b. The ECL intensity increased with an increased amount of GOD-labeled anti-Cry1Ac used for the construction of the immunocomplex and reached a plateau at 21 ng/mL. This result suggested that a sufficient amount of antibodies completely reacted with the antigens. Therefore, 21 ng/mL GOD-labeled anti-Cry1Ac was selected as the optimal concentration.

ECL Response to Cry1Ac. The calibration curve (Figure 7) for Cry1Ac detection using the prepared immunosensor under optimal conditions exhibited a linear range from 0 to 6 ng/mL, with a regression equation of $I = 520.81 + 1862.27C$ (ng/mL) and a correlation coefficient of $r = 0.9991$. The limit of detection (LOD) was 0.25 pg/mL (S/N = 3), indicating that the immunosensor for Cry1Ac detection was more sensitive than previously reported methods (Table 1).

Selectivity, Reproducibility, and Stability of the Cry1Ac Immunosensor. The effects of interfering substances, such as Cry1Aa, Cy1Ab, Cry2Ac, Cry1F, and Cry3Ac, which are similar in structure and protein properties to Cry1Ac, were used to verify the selectivity of the sensor. Cry1Ac-like Bt protein (including Cry1Aa, Cy1Ab, Cry2Ac, Cry1F, and Cry3Ac) were extracted from Bt HD-73 of soybean as described by Kain et al.[41] The extract was centrifuged for 10 min at 10 000 revolutions/min, and then Cry1Ac toxins were obtained after removing the upper layer. The concentration of Cry1Ac-like Bt protein was determined by the spectrophoto-

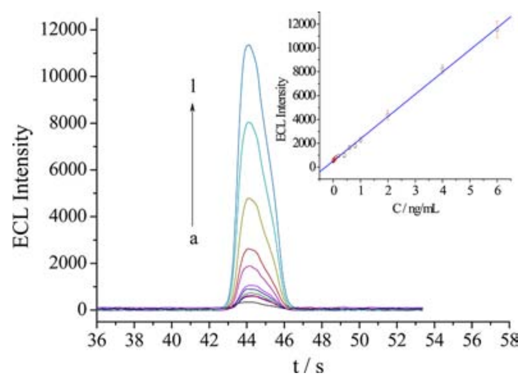

Figure 7. Response and calibration curves (inset) of Cry1Ac using the ECL immunosensor. The concentrations of Cry1Ac (a–l) were as follows: 0, 0.006, 0.01, 0.02, 0.06, 0.1, 0.2, 0.6, 1, 2, 4, and 6 ng/mL.

Table 1. Properties of Some Cry1Ac Detection Methods

number	detection methods	LOD	reference
1	immunomagnetic electrochemical sensor	0.05 ng/mL	15
2	ELISA	5 ng/mL	11
3	ELISA, Envirologix Cry1Ab/Cry1Ac plate kit	0.25 μg/mL	16
4	Abraxis Bt-Cry1Ab/Ac ELISA kit	0.125 ng/mL	17
5	biomimetic extraction and ELISA	11.6 ng/g	18
6	ELISA, Cry1Ac polyclonal kits	10 ng/mL	19
7	capillary electrokinetic chromatography	10 μg/mL	20
8	immuno-PCR	216 ng/mL	21
9	ECL, Fe_3O_4@Au NP membrane	0.25 pg/mL	proposed sensor

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Table 2. Analysis of Cry1Ac Concentrations in Clinical Sera Using the Proposed ECL Immunosensor

samples[a]	detected values by the proposed sensor	UV detected values for comparison (μg/mL)	relative standard deviation (n = 5)	Cry1Ac added to the samples (μg/mL)	total Cry1Ac found (μg/mL)	recovery (%)
1	19.52 μg/mL	19.43	0.59	5	24.76	101.4
2	25.17 μg/mL	25.18	1.68	5	30.96	102.6
3	26.45 μg/mL	26.59	0.93	5	31.38	99.34
4	26.89 μg/mL	27.12	1.32	5	32.13	100.0
5	30.23 μg/mL	30.40	1.79	5	35.99	101.7
6	1.43 ng/mL		0. 95	0.5	1.95	101.4
7	1.54 ng/mL		1.34	0.5	2.03	99.51

[a]Sample numbers 1−5 are the transgenic plant samples, and sample numbers 6 and 7 are the transgenic and non-transgenic plant composite samples.

metric method. The results demonstrated that at least 100 ng/mL Cry1Aa, Cy1Ab, Cry2Ac, Cry1F, and Cry3Ac did not affect the determination of 4 ng/mL Cry1Ac, with relative errors less than ±5%. Hence, the specific modified immunosensor was acceptable.

The regeneration of the Cry1Ac immunosensor was carried out by removing the magnet and rinsing all magnetic substances on the electrode. The electrode was then cleaned with 0.5 mol/L HCl solution. The reproducibility of the immunosensor was evaluated in several coherent experiments by determining the ECL response of 50 ng/mL Cry1Ac using five immunosensors fabricated with the same composite biomagnetic NPs. The result showed a relative standard deviation of 2.04%. A relative standard deviation of 4.03% was obtained for the same concentration of Cry1Ac for the five immunosensors constructed using different batches of biomagnetic NPs. This finding indicated the excellent reproducibility of the fabricated immunosensor.

The long-term stability of the immunosensor was determined in terms of the storage stability of biomagnetic NPs loaded with sandwich immune complexes, which was evaluated over a period of more than 45 days. To ensure stability, the bionanoparticles were refrigerated at 4 °C when not in use and applied for the measurement of 50 ng/mL Cry1Ac every 2−3 days. No obvious signal change was observed within 20 days. The ECL peak of the immunosensor changed by 6.84% compared to the initial response after 30 days and by 10% compared to the initial response after 45 days. This result indicated that the sensor had good long-term stability and the enzyme maintained its biological activity when immobilized onto the electrode.

Detection of Cry1Ac in Transgenic Bt Soybean Extract. To evaluate the analytical reliability and application potential of this immunosensor, transgenic Bt soybean extract was analyzed using the proposed method. The result was compared to the reference values obtained by the ultraviolet−visible (UV−vis) spectrophotometric method. Before determination, the samples of transgenic Bt soybean extract solution were diluted stepwise for 10^5 times, except for the transgenic and non-transgenic plant composite samples. The standard addition method was also used to check the accuracy of the results, which are listed in Table 2. An acceptable agreement with recoveries from 99.34 to 102.6% is shown.

In conclusion, a new and simple ECL immunosensor for transgenic Bt soybean Cry1Ac protein detection was designed on the basis of Fe_3O_4@Au paramagnetic NPs. Signal amplification was achieved because of the enzymatic reaction, analyte enrichment on magnetic capture, and sensitive ECL detection technique. The immunoassay exhibited a low detection limit, indicating that the proposed sensor is one of the most sensitive Cry1Ac sensors among previously reported sensors. The sensor can be used to distinguish trace transgenic Bt protein from a large background amount of non-transgenic plants. The sensor also showed great potential use for the reliable detection of transgenic products and can serve as a tool for the prevention of a mixture with genetically modified food. The immunosensor showed good stability because of the good biocompatibility and easy renewability of the gold-coated NPs. The strategy can be easily extended to other devices for bioanalytical and bioseparation applications.

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Notes

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