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ORIGINAL PAPER

Electrochemiluminescent aptamer biosensor for the determination of ochratoxin A at a gold-nanoparticles-modified gold electrode using *N*-(aminobutyl)-*N*-ethylisoluminol as a luminescent label

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Abstract A highly selective electrochemiluminescent biosensor for the detection of target nephrotoxic toxin, ochratoxin A (OTA), was developed using a DNA aptamer as the recognition element and *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) as the signal-producing compound. The electrochemiluminescent aptamer biosensor was fabricated by immobilizing aptamer complementary DNA 1 sequence onto the surface of a gold-nanoparticle (AuNP)-modified gold electrode. ABEI-labeled aptamer DNA 2 sequence hybridized to DNA 1 and was utilized as an electrochemiluminescent probe. A decreased electrochemiluminescence (ECL) signal was generated upon aptamer recognition of the target OTA, which induced the dissociation of DNA 2 (ABEI-labeled aptamer electrochemiluminescent probe) from DNA 1 and moved it far away from the electrode surface. Under the optimal conditions, the decreased ECL intensity was proportional to an OTA concentration ranging from 0.02 to 3.0 ngmL^{-1}, with a detection limit of 0.007 ngmL^{-1}. The relative standard deviation was 3.8% at 0.2 ngmL^{-1} (n=7). The proposed method has been applied to measure OTA in naturally contaminated wheat samples and validated by an official method. This work demonstrates the combination of a highly binding aptamer with a highly sensitive ECL technique to design an electrochemiluminescent biosensor, which is a very promising approach for the determination of small-molecule toxins.

Keywords Electrochemiluminescence · Aptamer · Ochratoxin A · Gold-nanoparticle-modified gold electrode

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Introduction

Ochratoxin A, *N*-[(3*R*)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)carbonyl]-L -phenylalanine; OTA (Fig. S1), is a mycotoxin that is produced by several species of the genera *Aspergillus* and *Penicillium* [1, 2] and occurs in a variety of food commodities, of which cereals (e.g., wheat, barley, corn, oats, and rye) are the most vulnerable. It has also been identified as a contaminant in coffee and wine throughout the world [3, 4]. OTA is a nephrotoxic toxin, with strong carcinogenic effects on rodents, as well as documented teratogenic and immunotoxic effects in humans [5, 6].

OTA contamination usually occurs in trace amounts ranging from nanograms to micrograms per gram of foodstuff. The published methods for the analysis of OTA are mainly chromatographic techniques, including thin-layer chromatography, gas chromatography, and high-pressure liquid chromatography (HPLC) [7–11]. Recently, capillary electrophoresis [12], solid-phase extraction–liquid chromatography–electrospray tandem mass spectrometry [13–15], liquid chromatography–tandem mass spectrometry [16], and solid-phase microextraction–liquid chromatography–fluorescence detection [17] were also reported.

Chromatographic methods generally require multiple steps prior to detection, including extraction, extensive sample cleanup, preconcentration, and sometimes derivatization of the analyte. To overcome the limitations encountered with the chromatographic analysis, immunoassay methods were developed [11, 18–21]. New immunosensor devices based on surface plasmon resonance, fiber optics, fluorescence polarization, and microbead-based assays have also been utilized for rapid mycotoxin analysis [22–26]. A new array biosensor based on immunoassay for the detection and quantitation of OTA was also reported recently by Ngundi et al. [27].

In recent years, biosensors for the detection of small molecular substances based on various analytical techniques, such as electrochemical [28], optical [29], and electrochemiluminescence (ECL) [30, 31] techniques, have attracted much attention. Among them, the ECL technique has many distinct advantages over the others because not only does it have a very high sensitivity but also simple formats are available for the ECL technique. Among the ECL techniques, tris(2,2′-bipyridyl) ruthenium(II) is the most widely used electrochemiluminescent label, and tripropylamine is the related coreactant [32–35]. Recently, *N*-(aminobutyl)-*N*-(ethylisoluminol) (ABEI), a luminol derivative, has been gradually used as an alternative electrochemiluminescent reagent. A promising property of this electrochemiluminescent reagent is the relatively high ECL efficiency even when it is chemically attached to specific analytes. Therefore, ABEI as a labeling reagent has been used in electrochemiluminescent biosensors [36, 37]. Some researches also have proved that ECL sensitivity can be greatly improved by using a gold nanoparticle (AuNP)-modified electrode by virtue of the catalysis of AuNPs [38, 39]. But to the best of our knowledge, there has been no report concerning the application of ABEI as a label in fabrication of an electrochemiluminescent aptamer biosensor.

Aptamers with advantages over many antibodies in that they are inexpensive, stable, and can be synthetically manufactured and chemically manipulated with relative ease have been shown to be useful as therapeutic agents and diagnostic tools [40]. Aptamers, which are single-stranded oligonucleotides generated using the iterative approach of systematic evolution of ligands by exponential enrichment [41] that can naturally fold into different three-dimensional structures, have the capability of binding specifically to a specific target [42–44]. If aptamers were used as affinity ligands instead of monoclonal and polyclonal antibodies, researchers would find a more promising alternative for mycotoxin capture and detection. Recently, for the first time, Cruz-Aguado and Penner [45] reported a DNA aptamer for selective OTA recognition and detection. They then applied it in a competitive fluorescence polarization assay for OTA detection and also used it as a sorbent for the preparation of affinity columns to extract OTA from wheat extracts [45, 46]. This opens a new way for the detection of small molecular mycotoxin.

In this work, ABEI labeling combined with AuNP amplification was implemented to develop a sensitive electrochemiluminescent aptamer biosensor for the determination of a small molecule. As a model system, OTA was chosen as an analyte and ABEI-binding aptamer was used as a molecular recognition element, which is more stable than the antibodies usually applied in immunoassays.

Experimental

Materials

A $HAuCl_4$ stock solution (2‰ $HAuCl_4$, w/w) was prepared by dissolving 1.0 g of $HAuCl_4{\cdot}4H_2O$ (Shanghai Reagent, China) in 412 mL of purified water and was stored at 4 °C. 1,3-Propanedithiol and ethanol were obtained from Shanghai Chemical Plant (Shanghai, China). OTA and ABEI were purchased from Sigma-Aldrich (USA). Distilled, deionized water was used throughout the analysis. Binding buffer [10 mM tris(hydroxymethyl)aminomethane, pH 8.5, 120 mM NaCl, 5 mM KCl, and 20 mM $CaCl_2$] was used for the binding reaction between the aptamer and OTA. Unless otherwise stated, all chemicals and reagents used in this study were of analytical-grade quality.

Complementary DNA 1 and DNA 2 synthesized by Shanghai Sangon Biological Science & Technology Company (Shanghai, China) were used to construct the biosensor recognition and indicator elements. SH-tailed DNA 1 is the complementary strand of DNA 2 and was immobilized onto the surface of a AuNP-modified gold electrode. DNA 2 is the OTA aptamer, reported by Cruz-Aguado and Penner [45]. NH_2-functionalized DNA 2 labeled with ABEI was employed to capture and bind OTA. The sequences were as follows:

DNA 1, 5′-TGT CCG ATG CTC CCT TTA CGC CAC CCA CAC CCG ATC-SH-3′

DNA 2, 5′-NH_2-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′

Apparatus

Figure 1 shows a schematic diagram of the ECL experimental setup of the aptamer biosensor system for OTA detection. The ECL experiments were performed with a CHI660B electrochemistry workstation (CH Instruments, Austin, TX, USA) for supply of the potential. All experiments were carried out with a three-electrode system. The working electrode was a AuNP-modified gold electrode. A

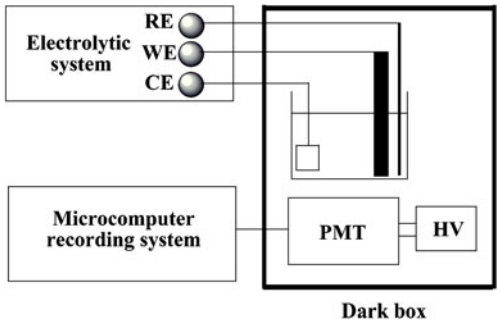

Fig. 1 Electrochemiluminescence (ECL) experimental setup of the aptamer biosensor system for ochratoxin A (OTA) detection. *WE* working electrode, *CE* counter electrode, *RE* reference electrode, *PMT* photomultiplier tube, *HV* high voltage

platinum wire was used as the counter electrode, and a silver wire was used as the reference electrode, which is invariable during the experimental period. All the potentials were measured and reported according to this reference electrode. The ECL intensity produced in the electrolytic cell was detected and recorded by an MPI-B chemiluminescence analyzer (Xi'an Remax Analytical Instrument Co., Xi'an, China), which was operated by a personal computer.

Stock and working standard solutions

A stock solution (0.1 $mg mL^{-1}$) of OTA was prepared by dissolving 1.0 mg OTA in 10.0 mL of ethanol and was kept in a brown volumetric flask. A 1.0-mL volume of this solution was further diluted to 10.0 mL with water to obtain a stock solution containing 10.0 $\mu g mL^{-1}$ of OTA. The working standard solution of OTA was prepared daily by serial dilution of the stock solution.

Preparation of the AuNP-modified electrode

AuNPs with a diameter of 10 nm were prepared by reducing $HAuCl_4$ with 1% trisodium citrate [39] and characterized using a transmission electron microscope (Hitachi H700, Japan) for the shape and size. Before modification, the bare gold electrode (d=3.0 mm) was polished with 1-, 0.3-, and 0.05-μm aluminum slurry on a polishing cloth, respectively, followed by thorough sonication in acetone and doubly distilled water. The electrode was then cleaned by cycling between 0 and 1.5 V versus the standard calomel electrode (SCE) in H_2SO_4 (0.5 M) at a scan rate of 100 $mV s^{-1}$ until reproducible cyclic voltammograms were obtained. It was further characterized by cycling between -0.2 and 0.6 V versus the SCE in phosphate-buffered saline (PBS; 0.1 M, pH 7.0) containing $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ (1:1) at a scan rate of 100 mV s^{-1} until a well-shaped cyclic voltammogram with a peak-to-peak voltage of 74 mV was observed. The pretreated bare gold electrode was immersed in a 2 mM 1,3-propanedithiol–ethanol solution, and was incubated at room temperature for 20 h. The thiol self-assembled monolayer on the surface of the gold electrode was rinsed with ethanol and ultrapure water. After that, the thiol-functionalized gold electrode was dipped into the gold colloid solution for 10 h at 4 °C. After it had been rinsedwith ultrapure water, the AuNP-modified electrode was allowed to dry at room temperature and was ready for further experiments. Five identically modified gold electrodes were used in this present work.

Preparation of the ABEI-labeled aptamer

ABEI was directly labeled onto the aptamer DNA with the well-established glutaraldehyde method [47, 48]. The labeling protocol was as follows:

1. ABEI (2 mg) was dissolved in hydrogen chloride solution and then PBS containing 5% glutaraldehyde was added. The reaction mixture was stirred for 2 h.
2. The aptamer DNA 2 was then added and further incubated with the mixed solution for 12 h at 4 °C with shaking.
3. The ABEI-labeled aptamer prepared was kept at 4 °C in PBS for further use.

ECL detection of OTA

A schematic illustration of the principle for OTA detection with the proposed electrochemiluminescent aptamer biosensor is presented in Fig. 2. In the absence of target, the ABEI-labeled aptamer probe is thought to hybridize with DNA 1 and be assembled onto the surface of gold electrode, resulting in a strong ECL signal owing to the tag being close to the electrode surface. In the presence of the target, the ABEI-labeled aptamer probe binds to OTA and is dissociated from the surface of electrode; thus a small ECL signal is generated owing to the decrease of the amount of ABEI-labeled aptamer probe assembled on the electrode surface.

First, an AuNP-modified gold electrode was prepared. Subsequently, the electrode was immersed in 1.0 mL of DNA 1 (2.0×10^{-6} M) for 4 h to form a DNA 1 self-assembled electrode and this was then washed twice with 0.1 M PBS. The resulting electrode was then immersed in 1.0 mL of DNA 2 solution for 30 min at 37 °C to form the complementary double strands of DNA 1–DNA 2. After that, the electrode was thoroughly washed with the same buffer. Then it was immersed in 500 μL of OTA solution for 30 min, followed by washing with binding buffer to remove the dissociating electrochemiluminescent probe on the electrode.

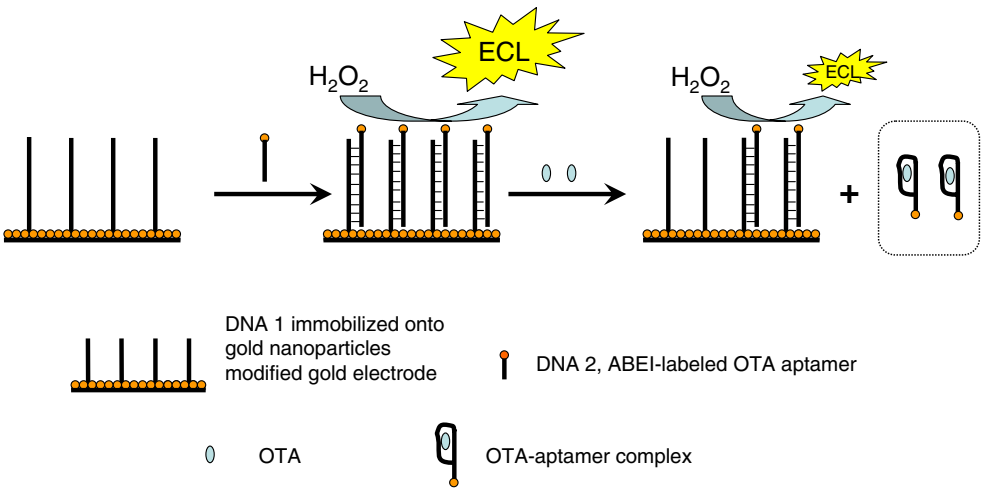

Fig. 2 Aptamer recognition and *N*-(4-aminobutyl)-*N*-ethylisoluminol (*ABEI*)-based biosensor for detection of OTA on a gold nanoparticle (AuNP)-modified gold electrode

ECL detection was performed at a constant potential of +0.80 V in 2.0 mL of buffer solution containing H_2O_2. The concentration of OTA was quantified by the decreased ECL signal.

Sample preparation and measurement

Twenty naturally contaminated wheat samples from six local markets of agricultural products were treated with an official method [49]. Briefly, each sample was ground by a high-speed disintegrator and passed through a test sieve (1-mm aperture). Twenty grams of each ground sample and 5 g NaCl were introduced into a 100-mL flask, the extracting solution (methanol+H_2O, 8:2) was added to the calibration line, the resultant solution was completely mixed and then transferred into the cup of a homogenizer, stirred at high-speed, and extracted for 2 min. Next, the resultant solution was filtered and 10.0 mL of filtrate was transferred into a 50-mL flask, water was added to the calibration tail and the solution was mixed for homogenization. The resultant solution was further filtered with glass-fiber filter paper until the filtrate was clear. The filtrate was collected in a clean container to measure OTA with the proposed electrochemiluminescent aptamer biosensor method as described earlier. For the standard addition and recovery experiment, the standard OTA was introduced into the wheat ground samples before extracting solution was added.

The filtrate was also treated and tested as follows to measure OTA with an official standard method [49]. A PuriToxSR O130 OTA immunoaffinity column (Beijing Rapidbio, Beijing, China) was connected to a 10-mL glass injector, and 10.0 mL of the filtrate described above was transferred into the injector. An air-pressure pump was then connected to the injector. The pressure of the pump was adjusted and the filtrate solution was allowed to pass through the immunoaffinity column at a rate of one drop per second until air entered in the column. Then 10 mL of OTA rinsing buffer solution (2.5 g NaCl, 5.0 g $NaHCO_3$, 0.1 mL Tween-20, diluted with water to 1 L) and 10 mL of water was used to wash the column in turn with a flow rate maintained at one to two drops per second. All the effluent was discarded, and the column was pumped and dried.

Subsequently, 1.0 mL of methanol was introduced into the affinity column at a rate of one drop per second to elute the bonded OTA. All the eluate was collected in a clean glass tube and was used for HPLC determination. An Agilent model 1220 HPLC system containing a fluorescence detector (Agilent, USA) was utilized to perform the measurements. The HPLC conditions used for the determination were as follows: the chromatographic column was a C_{18} column (5 μm, 150 mm×4.6 mm), acetonitrile–water–glacial acetic acid (99:99:2) was used as mobile phase, the flow was rate 0.9 $mL\,min^{-1}$, the column temperature was 35 °C, the sample injection amount was 10 μL, excitation was at 330 nm, and the fluorescence was detected at 477 nm. The OTA content was quantified from the fluorescence peak area.

Results and discussion

ECL behavior

ABEI-labeled DNA 2 was assembled onto the surface of the gold electrode through the hybridization of DNA 2 and DNA 1, and the binding of SH-functionalized DNA 1 and AuNPs immobilized onto the surface of the gold electrode. The ECL behavior of the assembled ABEI was investigated with cyclic voltammetry (CV) and a double-step pulse potential. It was found (Fig. 3) that the ECL signals of the assembled ABEI generated by a pulse potential were much stronger than those generated by CV. This might be

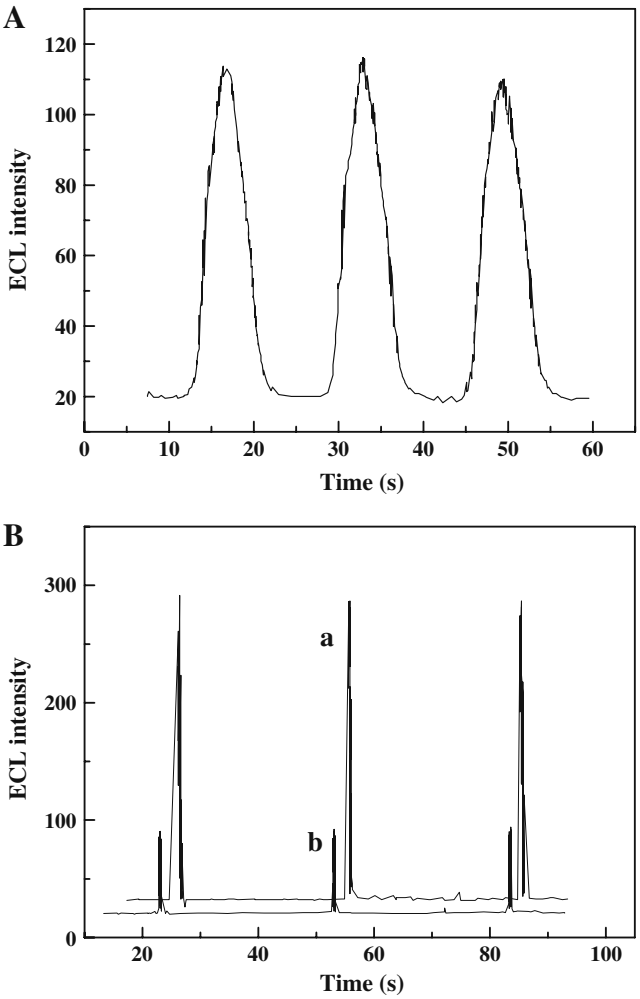

Fig. 3 **a** ECL signals under cyclic voltammetry, from 0 to 0.8 V, scan rate of 100 mVs^{-1}. **b** ECL signals under pulse potential. ECL signals were obtained on a AuNP-modified electrode (**a**) and on bare gold electrode combined with an ABEI-labeled aptamer (**b**). The potential of the ECL reaction was supplied by a CHI660B electrochemistry workstation, and the ECL signals were recorded by an MPI-B chemiluminescence analytical system

attributed to more ABEI molecules being oxidized under the pulse potential. Moreover, the ECL signals generated by the pulse potential were relatively stable for dozens of pulse potentials in each experiment, whereas the ECL signals generated by CV decreased with the increase of the cycling number. This may have resulted from the diffusion layer on the surface of electrode being difficult to recover in the next period under the CV. Therefore, pulse potential were adopted to produce the ECL signals in the following experiments. Furthermore, a tenfold amplification of the ECL of ABEI was observed with a AuNP-modified gold electrode, compared with that of with a bare gold electrode. This may have resulted from the catalysis of AuNPs on ABEI ECL [37, 50].

Optimization of ECL conditions

When a double-step potential was applied to the electrode, a strong pulse ECL signal could be achieved. To obtain highly sensitive ECL performance for the determination of OTA at the AuNP-modified electrode, the AuNP dose for gold electrode modification, the pH and medium, and the concentration of hydrogen peroxide were optimized.

Effect of potential

The ECL intensity was measured as a function of the pulse amplitude. The applied potential was therefore tested. It was found that almost no light emission occurred at potentials below 0.6 V, but above this value the light intensity increased sharply with potential up to 0.8 V, and reached a plateau between 0.8 and 1.0 V (Fig. S2). At higher potentials, a slightly decreased signal was observed, so 0.8 V was selected as the working potential.

Effect of pH

The ECL reaction of ABEI was greatly influenced by the pH of the solution (Fig. S3). The integral ECL intensity was very weak in acid or neutral solution, but increased from pH 7.0 to 11.0 and reached a maximum at pH 11.0. So pH 11.0 was used in the following experiments. The influences of the reaction media, including Na_2CO_3–$NaHCO_3$, KH_2PO_4–K_3PO_4, H_3BO_3–NaOH, and KCl–KOH, were further assessed for the ECL response. The results show that ABEI displayed the best response in Na_2CO_3–$NaHCO_3$ solution (pH 11.0).

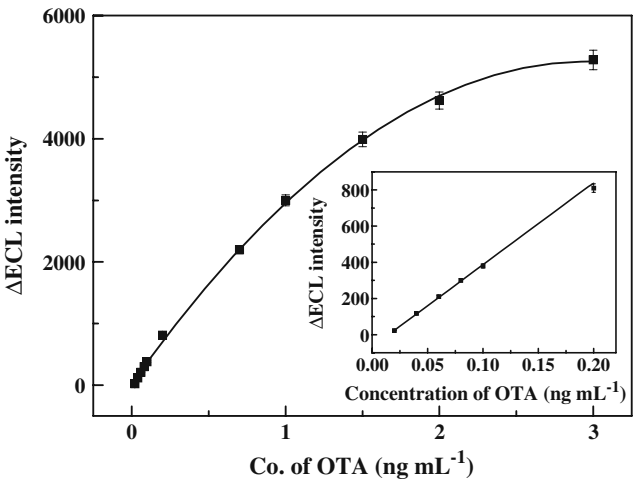

Fig. 4 Standard curve of the ECL intensity versus OTA concentration based on a AuNP-modified electrode combined with an ABEI-labeled aptamer

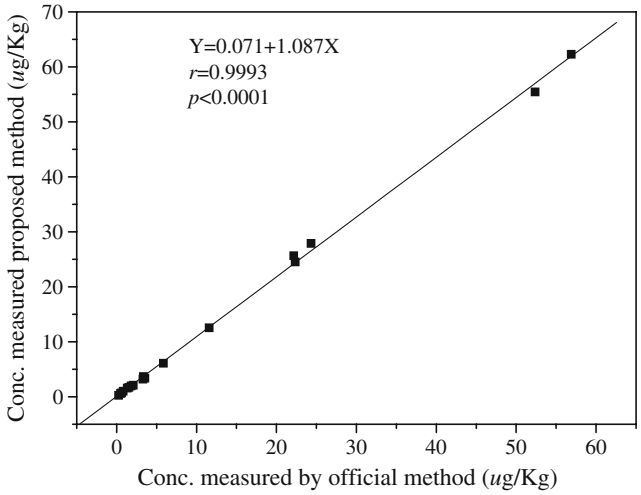

Fig. 5 Relationship between the proposed method and the official method for OTA detection in wheat samples. The data points are presented as the mean of five measurements

Effect of H_2O_2 concentration

The ECL reaction of ABEI in alkaline solution can be markedly improved by the addition of H_2O_2. It was observed (Fig. S4) that the ECL intensity increased with the increase of the H_2O_2 concentration from 0.01 to 1.0 mM, owing to an increase of the co-oxidizing function of H_2O_2, and reached a maximum at 1.5 mM. The blank ECL intensity was very high (data not shown) when the concentration of H_2O_2 was higher than 2.0 mM. Therefore, 1.5 mM H_2O_2 was chosen to improve the ECL response of ABEI.

Effect of dose of AuNPs

We found that AuNPs can effectively catalyze the ECL process and enhance the ECL response of ABEI. The influence of the dose of AuNPs on the ECL intensity was therefore investigated. The results indicated when less than 200 μL of AuNPs was used, the ECL intensity increased with increasing dose of AuNPs, which may be attributed to the fact that more AuNPs were immobilized on the surface of electrode. But when the dose of AuNPs was higher than 200 μL, the ECL intensity began to fall. We therefore choose 200 μL of AuNPs to modify the gold electrode.

Linear range, detection limit, and precision

Under the selected conditions, a nonlinear function was achieved for the ECL intensity and an OTA concentration range from 0.02 to 3.0 $ngmL^{-1}$ with the equation $\Delta I_{ECL} = -597.06C^2 + 3535.70C + 18.20$ (ΔI_{ECL} is the decrease in ECL intensity; C is the concentration of OTA in nanograms per milliliter; R^2=0.9993). The linear range for OTA was 0.02 to 0.2 $ngmL^{-1}$ ($\Delta I_{ECL} = 4347.90C - 55.66$, R^2= 0.9996), with a detection limit of 0.007 $ngmL^{-1}$ (3σ) (Fig. 4). The relative standard deviation was 3.8% for detecting 0.2 $ngmL^{-1}$ of OTA (n=7).

The response of the assembled ABEI–DNA system to OTA on a bare gold electrode was also examined as a control to validate the performance of the proposed electrochemiluminescent aptamer biosensor. The results (Fig. S5) show under the optimum conditions the bare gold electrode system could linearly sense OTA in the range 0.2 to 5.0 $ngmL^{-1}$, with a detection limit of 0.06 $ngmL^{-1}$, indicating the AuNP-modified gold electrode signal amplification technology gave an approximately a tenfold improvement in detection sensitivity compared with the bare gold electrode technology (we chose the lowest concentration of the linear range as the comparison standard).

Analytical application

The electrochemiluminescent aptamer biosensor was validated by using it to measure OTA in naturally contaminated wheat samples and comparing its performance with that of

Table 1 Recovery results for the added standard ochratoxin A (*OTA*) from wheat samples obtained by the proposed method

Sample	Initial OTA content (μg/kg)[a]	Added standard OTA content (μg/kg)	Detected content (μg/kg)[b] (mean±SD)	Recovery ratio (%)[c]
1	ND	0.5	0.41±0.22	82.0
2	ND	0.5	0.43±0.18	86.0
3	1.53	1.0	2.31±0.40	85.0
4	1.73	1.0	2.59±0.65	86.0
5	0.67	2.0	2.71±0.77	102.0
6	0.72	2.0	2.55±0.82	91.5
7	3.58	5.0	8.25±1.67	93.4
8	1.88	5.0	6.67±1.37	95.8
9	2.15	10.0	12.05±2.52	99.0
10	6.02	10.0	16.33±3.82	103.1

SD standard deviation, *ND* not detected

[a] Measured by the official standard method

[b] The average of five determinations

[c] Recovery ratio (%)=(detected content – initial OTA content)/added OTA content×100%

a current official standard method. As described in "Experimental," 20 naturally contaminated wheat samples from six local markets of agricultural products were treated and measured by the two analytical methods. Each sample was used for five parallel measurements to obtain the mean value,. The results show that the ECL determination of OTA in the samples generated concentrations of OTA not significantly different from those measured with the current official standard method ($p<0.0001$) (Fig. 5). This indicated the proposed electrochemiluminescent aptamer biosensor is accurate for the determination of OTA in real samples. Moreover, compared with the complicated procedures associated with the official standard method, such as extraction, concentration, elution, separation, and detection by a HPLC fluorescence detector, the proposed electrochemiluminescent aptamer biosensor offers the merits of simplicity, rapidity, and sensitivity.

The proposed electrochemiluminescent aptamer biosensor for OTA measurement in agricultural commodities was also evaluated by measuring the recovery of OTA by a standard OTA addition method, which involved the addition of a known quantity of OTA was added. As shown in Table 1, recoveries between 82.0 and 103.1% ($n=5$) were obtained for the proposed method, demonstrating the proposed biosensor can be applied to analysis of OTA in agricultural commodities.

In the present study, with the use of the affinity of an aptamer for OTA, we separated OTA from other grain components and determined OTA with an ECL method. The high specificity of the affinity of the aptamer for OTA allowed the direct determination of OTA by the ECL method with no need for other separation procedures. This work shows the application of aptamer recognition coupled with ECL detection for the quantitative analysis of OTA. An aptamer-based test for quantitative determination of a mycotoxin in food samples may open the way to a significant improvement in quality control and food safety with regard to more dynamic testing of agricultural products.

Conclusion

A novel electrochemiluminescent aptamer biosensor for OTA at a AuNP-modified gold electrode using an ABEI label was developed. An aptamer complementary DNA sequence was effectively immobilized on a AuNP-modified electrode, and aptamer tagged with ABEI labels was subsequently attached through DNA hybridization. The formation of double-stranded DNA produced excellent ECL responses to OTA under a double-step potential in alkaline solution. OTA in a concentration range from 0.02 to 3.0 ngmL^{-1} could be detected by the deceased ECL intensity. In the present method, the interferents in the food matrix can be well separated from OTA by simply washing the electrode without requiring multiple pretreatment processes, and thus the method was successfully applied for the detection of OTA in real samples. It is expected that it could be applied in the analysis of other toxins, biomolecules, and so on.

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