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Simply amplified electrochemical aptasensor of Ochratoxin A based on exonuclease-catalyzed target recycling

Ping Tong[a,b], Lan Zhang[a,*], Jing-Juan Xu[b,*], Hong-Yuan Chen[b]

[a] Ministry of Education Key Laboratory of Analysis and Detection for Food Safety, Analytical and Testing Center, The Sport Science Research Center, Fuzhou University, Fuzhou, Fujian 350002, China
[b] The State Key Lab of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing, Jiangsu 210093, China

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ABSTRACT

A new "signal-on" aptasensor for ultrasensitive detection of Ochratoxin A (OTA) in wheat starch was developed based on exonuclease-catalyzed target recycling. To construct the aptasensor, a ferrocene (Fc) labeled probe DNA (S1) was immobilized on a gold electrode (GE) via Au–S bonding for the following hybridization with the complementary OTA aptamer, with the labeled Fc on S1 far from the GE surface. In the presence of analyte OTA, the formation of aptamer-OTA complex would result in not only the dissociation of aptamer from the double-strand DNA but also the transformation of the probe DNA into a hairpin structure. Subsequently, the OTA could be liberated from the aptamer-OTA complex for analyte recycling due to the employment of exonuclease, which is a single-stranded DNA specific exonuclease to selectively digest the appointed DNA (aptamer). Owing to the labeled Fc in close proximity to the electrode surface caused by the formation of the hairpin DNA and to the analyte recycling, differential pulse voltammetry (DPV) signal could be produced with enhanced signal amplification. Based on this strategy, an ultrasensitive aptasensor for the detection of OTA could be exhibited with a wide linear range of 0.005–10.0 ng mL^{-1} with a low detection limit (LOD) of 1.0 pg mL^{-1} OTA (at 3σ). The fabricated biosensor was then applied for the measurement of OTA in real wheat starch sample and validated by ELISA method.

1. Introduction

Ochratoxin A (OTA) is one of the most common naturally produced mycotoxin mainly by *Aspergillus ochraceus*, *A. Carbonarius* and *Penicillium verrucosum* (Breitholtz et al., 1991). OTA has nephrotoxic, immunotoxic, teratogenic and carcinogenic effects (Bellí et al., 2007; Pohland et al., 1992), and exists in many food products including cereals, cacao, coffee beans, beer, grape juice and wine. For these reasons, the European commission has fixed maximum limits for OTA in wines and other foods, which is a maximum of 5 μg kg^{-1} for raw cereal grains and all derived products from cereal (Commission Regulation No. 1881/2006). Thus, the accurate monitoring of this mycotoxin in food products would be of highly importance.

The commonly used analytical techniques for the OTA detection includes thin-layer chromatography (TCL) (Saxena et al., 2001), high-performance liquid chromatography (HPLC) (Tessini et al., 2010), gas chromatography (GC), and their combination to fluorescence, ultraviolet, and mass spectrometry (MS) (Turner et al., 2009). However, these conventional methods usually necessitate expensive cost, time-consuming steps and need for specially trained personnel, which hindered their wide application (Ahmed et al., 2007). Enzyme-linked immunosorbent assays (ELISA) are also very popular due to the advantages of reliability and low requirement for equipment. Several immunoassay methods for the detection of OTA have been developed (Erkekoğlu et al., 2010; Liu et al., 2008). Regretfully, the antibody (Ab)-based immunoassays are still limited by high cost and low stability of Abs as well as the relative complexity of transducing antibody-antigen binding events. Hence the development of new advanced method for OTA bioanalysis is surely be desirable.

Aptamers are nucleic acid-based (DNA or RNA) affinity probes that provide a number of advantages over Abs including temperature stability, low cost and reusability (Ellington and Szostak, 1990). The specific binding affinity of this kind of artificial oligonucleotide for various targets, including small molecules (Sankaran et al., 2006), proteins (Dong et al., 2011), and even cells (Shangguan et al., 2006) make possible their wide biosensing applications. In general, each aptamer binds to one single target molecule. The aptamer of OTA was firstly obtained by Cruz-aguado in 2008 (Cruz-aguado and Penner, 2008). After that, several aptamer-based biosensors were demonstrated in the detection of OTA (Kuang et al., 2010; Wang

* Corresponding authors. Tel.: +86 25 83597294; fax: +86 25 83597294.
E-mail addresses: zlan@fzu.edu.cn (L. Zhang), xujj@nju.edu.cn (J.-J. Xu).

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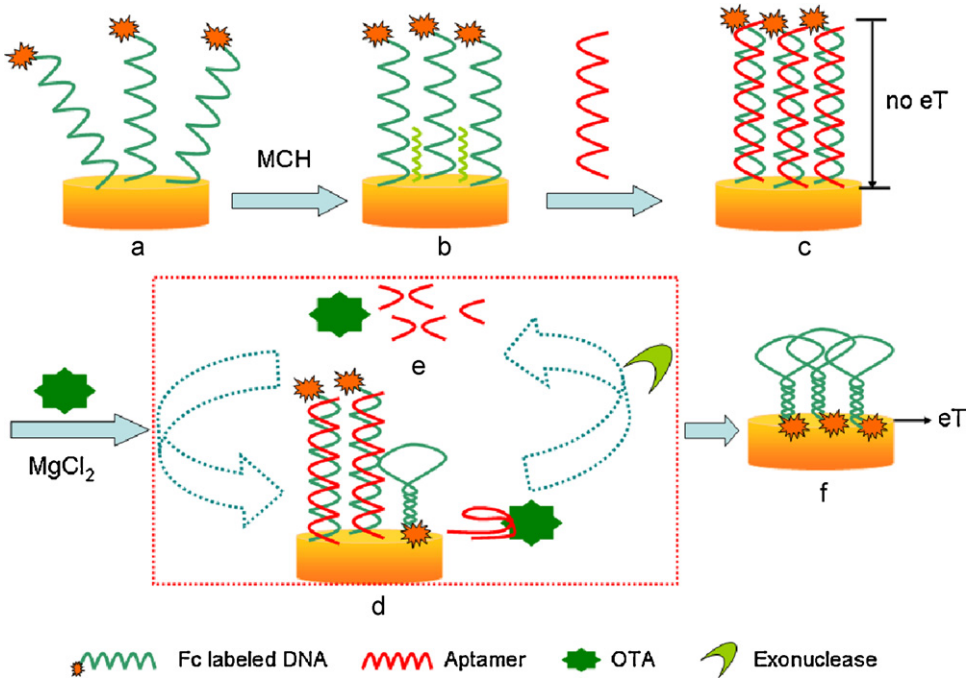

Fig. 1. Overview of the sensor scheme.

et al., 2010; Yang et al., 2011; Bonel et al., 2011; Barthelmebs et al., 2011; Prabhakar et al., 2011). However, the 1:1 binding ratio of the aptamer and OTA in these works limits the signal enhancement and the sensitivity of the assay (Lu et al., 2010).

Target recycling performed by enzymes has been demonstrated as an interesting and effective signal amplification route in some bioassay. For instance, DNAzymes (Sando et al., 2003), nicking enzymes (Chen et al., 2010) and exonucleases (Hsieh et al., 2010) have been applied in previous works of DNA biosensors. Nevertheless, the exploitation of the target recycling in the field of aptasensor is rather limited.

In this study, exonuclease-catalyzed target recycling strategy was applied for the first time to develop the electrochemical OTA aptasensor. The signal amplification approach was achieved by the formation of aptamer-OTA complex and the simultaneous release of OTA from aptamer-OTA complex by $RecJ_f$ exonuclease digestion. The quantification of OTA was completed by monitoring the signal variance generated from the labeled ferrocene (Fc) on the 5′ end of the probe DNA prior to and after the addition of OTA. The developed aptasensor exhibited wider linear range and lower detection limit than that of the existing OTA aptasensors. Furthermore, the content of OTA in real wheat starch sample was determined, the results were further validated by a commercial ELISA test with an excellent correlation between the two methods.

2. Materials and methods

2.1. Apparatus

Electrochemical measurements including cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) were performed on AutoLab electrochemical system (PGSTAT 302, Ecochemie, Netherlands). The detection was carried out in a common electrochemical cell containing a modified gold electrode (GE, i.d. 2 mm) as working electrode, a saturated calomel electrode (SCE) as reference electrode, and a platinum wire as auxiliary electrode. The mass spectrometry (MS) analysis was carried out by a LTQ-Orbitrap XL mass spectrometer (Thermofisher, USA).

2.2. Reagents

Ochratoxin A was purchased from Alexis Corporation (Lausen, Switzerland.). DNA 1 (Fc labeled probe DNA, S1): 5′-Fc–CCG ATG CTC CCT TTA CGC CAC CCA CAC CCG ATC GG-$(CH_2)_6$-SH-3′; DNA 2 (DNA aptamer, S2): 5′-AAA GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′. In this experiment, five pairs of complementary bases (underline showed) was designed at the both ends of the probe DNA to ensure the finally formation of hairpin structure. In addition, three bases were added at the 5′ end of the OTA aptamer for the purpose of making $RecJ_f$ exonuclease easier recognize the single strand in aptamer-OTA complex. Oligonucleotides were synthesized by TaKaRa biotechnology Co., Ltd. (Dalian, China). $RecJ_f$ exonuclease was obtained from New England Biolabs (Beijing) Ltd. (Beijing, China). 6-Mercapto-1-hexanol (MCH) was purchased from Acros organics. Enzyme-linked Immunosorbentassay Kit of OTA was bought from Huaan Magnech Bio-Tech Co., Ltd. (Beijing, China). Deionized water supplied by a Milli-Q water purifying system was used to prepare buffer, standard solution and sample preparation solution.

2.3. Aptasensor fabrication

The whole fabrication process of this biosensor is shown in Fig. 1. A GE was firstly polished to obtain mirror surface with 0.05 μm alumina powder, followed by sonication in ethanol and water for 5 min, respectively. Then the GE was cleaned by electrochemical etching in 0.5 mol L^{-1} H_2SO_4 using cyclic voltammetry from −0.3 V to 1.5 V versus SCE reference electrode at a scan rate of 100 mV s^{-1} for 50 scans. After drying with nitrogen, the electrode was immediately used for DNA immobilization. Firstly, 6 μL of 1.0 μmol L^{-1} S1 solution was first coated on the pre-cleaned gold electrode surface for 12 h (Fig. 1a). Next, this electrode was immersed in 1 mmol L^{-1} MCH for 1 h to remove the nonspecifically adsorbed DNA and make the probe DNA orientation to favor hybridization (Fig. 1b). Then,

6 μL of hybridization buffer containing 1.0 μmol L^{-1} S2 was spread on the resulted GE. The hybridization was carried out for 2 h at 37 °C. Afterwards, the sensor was then immersed in 150 μL solution of OTA and $RecJ_f$ exonuclease (0.03 U μL^{-1}) in 1× NEBuffer 2 (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) for 90 min and rinsed with water finally (Fig. 1d).

2.4. Gel electrophoresis

A Western Electrophoresis apparatus (BIO – RAD, USA) was applied for the polyacrylamide electrophoresis analysis. Polyacrylamide gel was prepared by mixing 6.66 mL 36% bis-acrylamide, 50.0 μL 12.5% ammonium persulfate and 10.0 μL TEMED in 20 mL 1× TBE (0.01 mol L^{-1} Tris borate–EDTA buffer) containing 2.8 mol L^{-1} urea. Then 1.0 mL fresh prepared polyacrylamide gel was transferred to the electophoretic system, and gelation for 30 min. Then 8.0 μL 5.0 μmol L^{-1} DNA was introduced in the gel, and a double-stranded DNA ladder from 600 bp to 100 bp (KeyGEN Biotech, Nanjing, China) was placed in as marker simultaneously. The electrophoresis was performed by voltages of 100 V for 1.5 h. After dyed by ethidium bromide (EB), electrophoresis image was obtained by photographed under UV light.

2.5. Electrochemical detection

In this experiment, CV was carried out at a scan rate of 100 mV s^{-1}. DPVs were performed in 10 mmol L^{-1} PBS buffer solution (pH 7.4) the potential interval +0.1–+0.6 V (vs. SCE), under the following conditions: modulation amplitude 0.05 V, pulse width 0.06 s, and sample width 0.02 s. Electrochemical impedance experiments were completed in the presence of 5.0 mmol L^{-1} (1:1) $[Fe(CN)_6]^{3-/4-}$, the biased potential was 0.20 V (vs. SCE), the amplitude was 5.0 mV, and the electrochemical impedance spectra were recorded in the frequency range of 100 kHz to 0.1 Hz with a sampling rate of 12 points per decade. A Nyquist plot (Zre vs. Zim) was drawn to analyze the impedance results.

2.6. Sample preparation

After kept in warm and humid environment for 30 days, 1.0 g wheat starch sample were extracted with 5.0 mL of acetonitrile–water solution (v/v = 6/4) by shaking on an orbital shaker for 20 min at room temperature. Extracting solutions were centrifugated (10 min, 4000 rpm), and then 1 mL of the supernatants was mixed with 1 mL of 10 mmol L^{-1} PBS buffer (pH 7.4) for the aptasensor detection.

2.7. MS analysis

The wheat starch extract was identified by electrospray ionization mass spectrometry (ESI-MS). The mass spectrometer was operated in the positive ESI mode. Nitrogen was used as the nebulizer and the drying gas pressure was set at 30 psi at the temperature of 350 °C. Capillary voltage was 3.5 kV. The mass range was set in the range of 100–600 *m*/*z*.

3. Results and discussion

3.1. Design strategy of the aptasensor

An electrochemical sensing interface for highly sensitive detection of OTA based on $RecJ_f$ exonuclease target-catalyzed recycling was proposed. The assay protocol of the electrochemical aptasensor was depicted schematically in Fig. 1. The surface of GE was first modified with the S1 (6 μL, 1.0 μmol L^{-1}) via Au–S bonding (Fig. 1a). After 6 μL, 1.0 μmol L^{-1} S2 hybridized to the S1, the double-strand oligonucleotides (dsDNA) were fabricated. So the redox moiety labeled on the S1 was kept relatively far from the surface of the GE, and the electron transfer (eT) essentially retarded as shown in Fig. 1c. When OTA was introduced, the S2 preferred to form the aptamer-OTA complex, which resulted in the dehybridization and released into the solution (Fig. 1d). And then the single S1 on the GE surface with tailor-made complementary bases at both ends could form a stable hairpin structure in the presence of Mg^{2+}, and as a consequence, the eT upon of the Fc was facilitated due to the close contact between the Fc and the GE (Fig. 1d). At the same time, the $RecJ_f$ exonuclease selectively degraded single-stranded DNA (ssDNA) S2 in aptamer-OTA complex in the direction of 5′→3′, with the digestion terminating after the ssDNA was fully consumed. In this way, OTA was released (Fig. 1e) and was able to trigger the permanent transformation of multiple S2 from the dsDNA to aptamer-OTA complex, thereby generate an amplified faradic current (Fig. 1f). On the basis of this, a sensitive "signal-on" electrochemical aptasensor was established by monitoring the increase of DPV current.

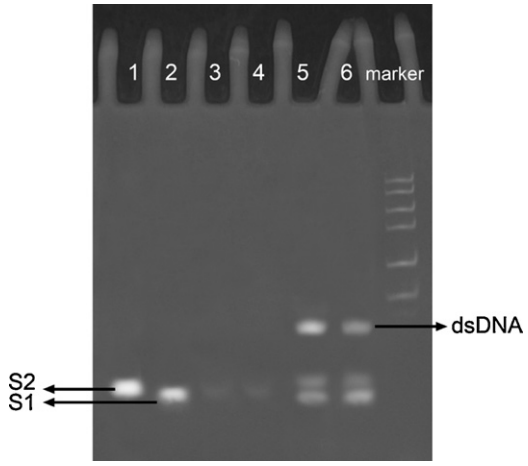

Fig. 2. Polyacrylamide gel electrophoresis of S2 (lan 1), S1 digestion product (lan 2), S2 digestion product (lan 3), aptamer-OTA complex digestion product (lan 4) and dsDNA digestion product without (lan 5) or with (lan 6) OTA.

Polyacrylamide gel electrophoresis was applied to verify the strategy. Polyacrylamide gel electrophoresis of S2 (lan 1), S1 digestion product (lan 2), S2 digestion product (lan 3), aptamer-OTA complex digestion product (lan 4), dsDNA digestion product without (lan 5) and with (lan 6) OTA was studied. The exonuclease digestion products were obtained by 2 h of exonuclease incubation and following 10 min of exonuclease inactivation (heating at 65 °C). As shown in Fig. 2, one distinct band was presented in lan 1 and lan 2. The band in lan 2 illustrated the existence of S1 even after it's incubation with exonuclease, since S1 can be protected from cleavage due to the stable hairpin structure of S1 in the presence of Mg^{2+} as well as the Fc group labeled in 5′ end. In addition, barely noticeable band was shown in lan 3 and lan 4, which indicated that the S2 was no more presence because of being digested by exonuclease. In lan 5 and 6, three bands appeared, suggesting the existence of dsDNA (the first band), whereas, another two bands were S1 and S2 which derived from the denaturation of dsDNA during the course of gel electrophoresis. This verified that the dsDNA could avoid the digestion from $RecJ_f$ exonuclease. Thus, the polyacrylamide gel electrophoresis exhibited that $RecJ_f$ exonuclease could degrade S2 even in aptamer-OTA complex but not Fc labeled probe DNA and dsDNA.

3.2. Study of the electrochemical behaviors at the modified electrode surface

Fig. s1A displays the EIS of various modified electrodes in 5.0 mmol L^{-1} (1:1) $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol L^{-1} KCl. For the bare GE, very small charge-transfer resistance (R_{ct}), which was measured as the radius of the semicircle in the EIS (curve a), suggesting a fast electron-transfer process at such electrode. Compared with the bare GE, the S1 modified GE showed a larger R_{ct} (curve b), mainly owning to the enlarged charge-transfer distance caused by the electrostatic repelling between the $[Fe(CN)_6]^{3-/4-}$ redox couple and the negatively charged S1 confined onto the GE. The R_{ct} was further enlarged after the S1 modified GE was blocked with MCH, as a result of inhibited electron transfer kinetics of MCH on the electrode (curve c). R_{ct} reached the maximum value when the OTA aptamer hybridized to the S1 (curve d), suggesting the formation of dsDNA on the electrode surface. After the dsDNA modified GE was incubated in OTA solution containing $RecJ_f$ exonuclease, the R_{ct} decreased greatly (curve e), this attributing to the fact that the formation of aptamer-OTA complex led to the dehybridization and release into the solution. These results were in a good agreement with the CV results (see Fig. s1B). Thus, hybridization and dehybridization events in this strategy were both confirmed.

3.3. Optimization of aptasensor preparation conditions

In the process of electrochemical aptasensor preparation, the immobilization of probe DNA on the electrode surface is a crucial step because densities of probe DNA directly affect the performance of the sensor. The immobilization time ranging from 3 to 18 h was investigated. As shown in Fig. s2A, it was clearly observed that the peak current increased greatly with the increase of immobilization time when the time was less than 12 h, and then the peak current increased only a little or even decreased with the increase of immobilization time. Thus, 12 h was applied in this experiment for the probe DNA immobilization.

Another fact that can impact the sensor sensitivity is the incubation time, which was closely related to the formation of aptamer-OTA complex as well as the $RecJ_f$ exonuclease-catalyzed target recycle. Fig. s2B displayed that the peak current increased with the increment of incubation time from 20 to 90 min, and then leveled off. Therefore, 90 min was selected as the incubation time for the detection of OTA in this study.

3.4. Performance of the exonuclease-catalyzed aptasensor

Under the selected experimental conditions, the sensitivity of the electrochemical aptasensor was investigated by DPV. The inset of Fig. 3 displayed typical DPVs after the dsDNA/MCH/ssDNA modified gold electrodes were incubated in different concentrations of OTA aqueous solutions containing 0.03 U μL^{-1} $RecJ_f$ exonuclease and 1X NEBuffer 2 (50 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris–HCl, 10 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} DTT, pH 7.9). The evident peak at approximately +0.37 V showed the reduction of the Fc moiety on the electrode surface. And the peak currents were increased with increasing the OTA concentrations in incubation solution and were linear with the OTA concentration in the range of 0.005–10.0 ng mL^{-1} shown in Fig. 3 (line a). The linear regression equation was adjusted to $\Delta I(nA) = 1.666\,C\ (ng\,mL^{-1}) + 5.298$ ($R = 0.9990$) with a detection limit of 1.0 pg mL^{-1} estimated by 3σ. As a comparison, a general aptasensor preparing under the same conditions but without $RecJ_f$ exonuclease was also studied, and the working curve with less sensitivity was shown in Fig. 3 (line b). Furthermore, the DPVs of exonuclease-catalyzed aptasensor and general aptasensor after incubation in 5 ng mL^{-1} OTA were shown in Fig. s3, it could be found that the current response of exonuclease-catalyzed aptasensor showed great enhancement compared with the general one. These experimental results, to some extent, verified the amplification strategy of exonuclease-catalyzed target recycling.

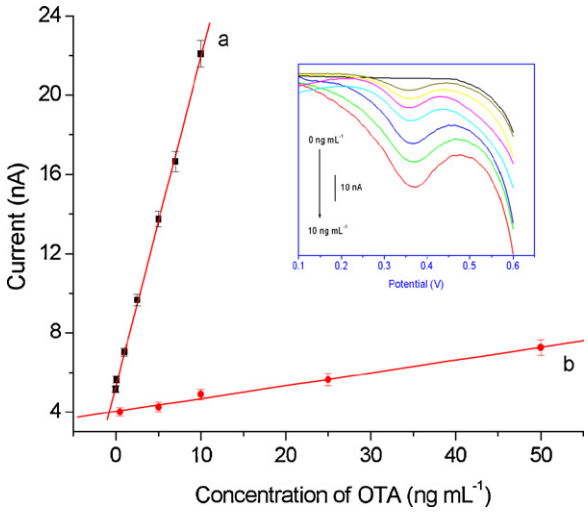

Fig. 3. The plot of the peak current of Fc as a function of OTA concentration with and without exonuclease. *Inset*: DPV of Fc moiety on the aptasensor surface after its incubation with 0, 0.005, 0.1, 1, 2.5, 5, 7, 10 ng mL^{-1} of OTA. Basic liquid: 10 mmol L^{-1} PBS buffer solution (pH 7.4).

A comparison of linear ranges and detection limits of existing aptasensors as well as our aptasensor for OTA detection is shown in Table 1. It was found that our exonuclease-catalyzed aptasensor exhibited wider linear range and lower detection limit than the previous reported aptasensors (Kuang et al., 2010; Wang et al., 2010; Yang et al., 2011; Bonel et al., 2011; Barthelmebs et al., 2011).

3.5. Specificity of the aptasensor for the detection of OTA

The specificity of the developed aptasensor was tested by the incubation reaction of dsDNA on the aptasensor surface with different targets, such as crude toxin microcystin-LR (MC-LR), thrombin and cocaine which were studied extensively with regard to aptasensors. As shown in Fig. 4, the peak currents of the blank, thrombin (10 ng mL^{-1}), cocaine (10 ng mL^{-1}), MC-LR (10 ng mL^{-1}) and OTA (6.0 ng mL^{-1}) were 0.7477, 1.927, 1.699, 1.952 and 14.999 nA, respectively. It clearly showed that the peak current of OTA was significantly higher than that of other

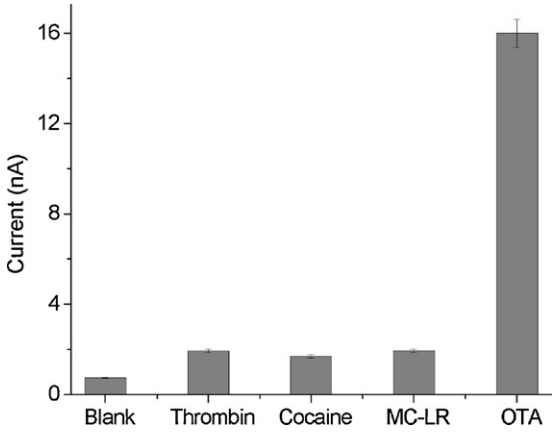

Fig. 4. DPV current of aptasensor after incubation with different targets. Experimental conditions are as Fig. 3.

Table 1
Comparison of linear ranges and detection limits of various aptasensors related to OTA.

No	Method	Linear range ($ng\,mL^{-1}$)	Detection limit ($pg\,mL^{-1}$)	Reference
1	Electrochemical aptasensor	0.1–20	30	Kuang et al. (2010)
2	Electrochemi-luminescent aptasensor	0.02–3.0	7	Wang et al. (2010)
3	Colorimetric biosensor	8060–251,875	8060	Yang et al. (2011)
4	Electrochemical aptasensor	0.78–8.74	70	Bonel et al. (2011)
5	Electrochemical aptasensor	0.11–15	110	Barthelmebs et al. (2011)
6	Exonuclease-catalyzed electrochemical aptasensor	0.005–10.0	1.0	This work

Table 2
Detection results for OTA in wheat starch samples obtained by the aptasensor and ELISA as reference method.

Sample	Sample no.	Dose of the spiked OTA ($ng\,mL^{-1}$)	Methods (mean ± SD, $ng\,mL^{-1}$) and recovery (%)	
			Aptasensor	ELISA
Wheat Starch	1	0	0.18 ± 0.10 (–)	0.21 ± 0.08 (–)
	2	0.1	0.27 ± 0.09 (90.0%)	0.32 ± 0.11 (110%)
	3	0.5	0.71 ± 0.12 (106%)	0.78 ± 0.13 (115%)
	4	1.0	1.26 ± 0.19 (108%)	1.15 ± 0.23 (94.1%)

analytes, suggesting the high specificity between OTA aptamer and OTA.

3.6. Evaluation of real samples and intralaboratory validation

OTA is a metabolin produced by genus *Aspergillus* and *Penicillium*, it can be found in the moldy foods. In this experiment, a mildewed wheat starch sample was studied. The sample preparation was performed by the extraction method mentioned in Section 2.6. In order to verify whether the wheat starch extract contained OTA, the extract was firstly determined by ESI-MS, and the ESI mass spectrum was shown in Fig. s4. The $m/z = 403.14$ was found corresponding to molecular mass of OTA, and then because the ESI-MS was performed in the positive ionization mode, the fragment $[M+H]^+$ with an $m/z = 404.14$ was also monitored. Therefore, OTA existing in the wheat starch sample was identified by ESI-MS.

To quantify the content of OTA in the wheat starch extract, it was detected by the developed exonuclease-catalyzed aptasensor. In addition, the aptasensor was also evaluated by investigating the recovery of the method through a standard addition method. The recovery experiment was carried out by adding three levels of OTA standard solution into the wheat starch extract and calculating the concentration from the linear regression equation. From the ratio between found amount and added amount, the recovery values were obtained. The DPV current of wheat starch samples is shown in Fig. s5. The experimental results were summarized in Table 2. The content of OTA in the wheat starch sample was detected as $0.36\,ng\,mL^{-1}$, and the recovery in wheat starch sample is 90.0–108%. The detection results were confirmed by using the commercially available ELISA as reference method shown in Table 2.

4. Conclusions

The work introduced a new exonuclease-catalyzed target recycling strategy to achieve amplified electrochemical aptasensing of OTA. Due to the introduction of the exonuclease into the system for analyte recycling, excellent sensitivity could be obtained through the proposed protocol. The developed biosensor was successfully applied to wheat starch sample detection, and the experimental results showed good agreement with those of the commercial ELISA method. This new method exhibits the advantages of simple, low cost and high sensitivity; it is potentially attractive for the analysis of OTA in food in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.07.075.

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