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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.5b03314 • Publication Date (Web): 06 Nov 2015

Exonuclease-Catalyzed Target Recycling Amplification and Immobilization Free Electrochemical Aptasensor

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Abstract

A simple,sensitive and selective immobilization free electrochemical aptasensor had been developed which combines the advantages of the discrimination of the aggregation of long and short DNA on negative charged indium tin oxide (ITO) electrode, high selectivity of the aptamer and high efficiency of exonuclease-catalyzed target recycling amplification. Ochratoxin A (OTA), a type of mycotoxin, has been chosen as the model target. Methylene blue (MB) labeled probe DNA had been hybridized with the OTA aptamer first, which cannot diffuse freely to the negative charged ITO electrode surface due to the repulsion of the negative charges since the hybridized DNA contains large negative charges. At present of target(OTA), the aptamer prefers to form OTA-aptamer complex in lieu of aptamer-DNA duplex, which results in the dissociation of probe DNA from the probe DNA/aptamer complex. The released probe DNA could be digested into mononucleotides including a MB-labeled electroactive mononucleotide (eT) due to the employment of the $RecJ_f$ exonuclease, a single-stranded DNA specific exonuclease. Since the eT contains little negative charge, which can diffuse easily to the negative charged ITO electrode surface and results in the enhanced electrochemical response detected. At the same time, the aptamer in the OTA-aptamer complex can be digested by $RecJ_f$ exonuclease also to liberate the target, which can participate in the next reaction cycling and realize the electrochemical signal amplification. Based on this strategy, an ultrasensitive homogeneous immobilization free electrochemical aptasensor for OTA can be developed with a low detection limit (LOD) of 0.004 ng mL^{-1} (S/N=3). The

proposed biosensor combines the advantage of the simple of immobilization free homogeneous ITO based electrochemical determination, high efficiency of exonuclease-catalyzed target recycling and high selectivity of the aptamer. The fabricated biosensor has been applied to detect OTA in real samples with satisfied results. The same strategy can be applied to develop biosensor for diverse targets.

Keywords: homogeneous, electrochemical aptasensor, signal amplification, ITO electrode, target recycling strategy, Ochratoxin A.

Aptamers are oligonucleotides which can bind specific targets with high affinity and specificity. Aptamers have the outstanding properties such as good temperature stability, low cost, reusability and easy modification.[1-5] Electrochemical detection technique has the characters of low-cost, rapidity, high sensitivity and amenability of on-field application, which have been coupled with aptamers to develop sensitive and selective biosensors for versatile targets.[6-8] Since the binding ratio of the aptamer and the target is 1:1, each aptamer can only bind with one single target molecule, so the detection limit is not low enough. In order to push down the detection limit of the aptasensor, various signal amplification strategies have been proposed.[9-10] The targets in these assays can be recycled and produce multiple signals.[11] Variety of nucleases, such as endonuclease[12] and exonuclease[13], have been employed as the cleavage enzymes in the amplification assays. $RecJ_f$ exonuclease is a single-stranded DNA (ssDNA) specific exonuclease which can catalyze the removal of deoxy-nucleotide monophosphates from DNA in the 5' → 3' direction. It can also catalyze the stepwise removal of the DNA strand of aptamer-target complex from 5'-terminus. This character has been applied to develop many sensitive aptasensors. Tong et al reported a sensitive electrochemical aptasensor for Ochratoxin A (OTA) with the assistant of $RecJ_f$ exonuclease digestion.[14] Jiang et al showed a exonuclease-catalyzed target recycling and DNA concatemers based aptasensing platform for simultaneous detection of thrombin and OTA.[15] Yi et al reported a similar electrochemical aptasensor for thrombin determination.[16] These biosensors indicated that high amplification efficiency can be achieved with the assistant of $RecJ_f$

exonuclease-catalyzed, but all these sensors need the immobilization of probe DNA on the electrode surface and the hybridization of DNA on the electrode/solution interface. The immobilization procedures are tedious and time consuming since which often involve the multistep surface-modified processe of electrode. Compared with homogeneous assays, the hybridization of the DNA and the recognition of the aptamer by the target in these sensors occur on the electrode/solution interface, the spatial hindrance effect of the electrode surface and the loss of configurational freedom of the aptamer usually make these heterogeneous assays suffer from relatively low binding and recognition efficiencies.[17] So it is necessary to find out some way to across these drawbacks.

DNA contains negative charge due to the negatively charged phosphate on molecular skeleton. Short DNA contains few negative charges while long DNA contains large negative charge. Compared with long DNA, short DNA can diffuse much more easily to the negatively charged Indium Tin Oxide (ITO) electrode surface since the repulsion is much smaller, which had been applied to develop immobilization free homogeneous electrochemical biosensors.[18,19] The DNA hybridization and target recognizing processes of these sensors all occur in the homogeneous solution phase, producing the advantages of simple operation, rapid response and improved reaction efficiency. However, because of the diffusion-controlled nature results, which refers to the electrochemical response of immobilization-free electrochemical depends on the diffusion of electro-active molecules from the solution phase to the surface of the electrode, the

immobilization-free electrochemical method has a lower sensitivity compared to the immobilization-required assays in which the electro-active molecules are attached or adsorbed on the electrode surface.[17] Therefore, many high efficiency signal amplification strategies had been coupled with this technique to enhance the sensitivity of the sensors. Liu et al proposed immobilization-free homogeneous electrochemical biosensor for telomerase detection via T7 exonuclease-catalyzed target recycling amplification.[20] But a variety of enzymes (T7 exonuclease and telomerase) used at the same time leads to the needing of rigorous conditions. I-Ming Hsing et al showed an homogeneous electrochemical biosensor for DNA detection combined with exonuclease III enhanced signal amplification tactics.[21] But the selectivity of the amplification assay utilizing a DNA polymerase is lower. In an early study, we reported an ultraselective homogeneous DNA electrochemical biosensor based on nicking endonuclease signal amplification (NESA), which exhibits a high distinction ability to single-base mismatch and double-bases mismatch.[22] But although the limitation of the biosensor will be no longer a problem with more and more nicking enzymes available commercially, it can only be applied to the DNA sequence containing the recognition site of the nicking endonuclease. Liu et al developed a homogeneous electrochemical ATP assay based exonuclease III assisted target recycling.[23] However, the intricate hairpin-aptamer probe need to be designed first, the re-organized G-quadruplex hairpin structure formation is hard, and the enzymatic cleavage activity of exonuclease III might be more accurate.

Ochratoxin A (OTA) is a common naturally produced mycotoxin,[24] which was

classified as a possible human carcinogen by the International Agency for Research on Cancer.[25] So it is necessary to find out some simple but sensitive and selective ways for OTA determination. Till now, lots of sensitive and selective electrochemical aptasensors for OTA determination had been reported, [26-27] but as mentioned above, nearly all these sensors need probes immobilization on the electrode surface and DNA hybridization at the electrode/solution interface. In this study, a simple, sensitive and selective homogeneous electrochemical OTA aptasensor had been designed that aims to prove the potential breakthroughs of integrating immobilization free electrochemical with the high selectivity of aptamer. A methylene blue (MB) labeled probe DNA had been hybridized with the OTA aptamer to form a double-stranded DNA (dsDNA) first. The conformational change upon OTA binding can resolve dsDNA into a single-stranded DNA (probe DNA) and an aptamer-OTA complex. Furthermore, the probe DNA and the aptamer in aptamer-OTA complex can be digested by the $RecJ_f$ exonuclease, producing a MB-labeled electroactive mononucleotide (eT), which can diffuse easily to the ITO electrode surface and results in the enhanced electrochemical response detected. The liberated OTA can bind with its aptamer in the dsDNA again to initiate the next generation to realize the signal amplification. By this way, a sensitive but simple electrochemical aptasensor can be developed for OTA determiantion.

EXPERIMENTAL SECTION

Reagents. All oligonucleotides were synthesized by TaKaRa biotechnology Co., Ltd.

(Dalian, China). Their sequences are shown below:

Methylene blue (MB) labeled probe DNA: 5'-AAACCGATGCTCCCTTTACGCCACCCACACCCGATC-MB-3'; OTA aptamer: 5'-AAAGATCGGGTGTGGGTGGCGTAA AGGGAGCATCGGACA-3'

Three additional bases had been added at the 5 end of the OTA aptamer for the purpose easier recognization of the single strand by the RecJf exonuclease in aptamer-OTA complex. $RecJ_f$ exonuclease and 10× NEBuffer 2 (50 mM NaCl, 10 mM Tris–HCl, 10 mM MgCl2, 1 mM DTT, pH 7.9) were obtained from New England Biolabs (Beijing) Ltd. (Beijing, China). OTA, Ochratoxin B (OTB), Ochratoxin C (OTC) and Aflatoxin B1 (AFB1) were purchased from Sigma Aldrich Chemical Co. Ltd.(St. Louis, MO) and used directly without further purification. SYBR Green I (10000×) was purchased from Xiamen Biovision Biotechnology Co. Ltd. (Xiamen, China). The quality control corn samples were purchased from Clover Technology Group, Inc., (Beijing, China). Other chemicals were of analytical reagent grade. All solutions were prepared with deionized water (Milli-Q, Millipore, resistance 18.2 MΩ).

Electrochemical Detection System. Differential pulse voltammetry (DPV) signal was measured on a CHI660a electrochemical system (CH Instruments, Shanghai, China). The electrochemical detection system contains an ITO working electrode, a Pt reference electrode and a Pt counter electrode. The potential of the Pt reference electrode in the buffer was determined to be +0.36 V with respect to an Ag/AgCl reference electrode. The negatively charged ITO working electrode surface was

reached through the following treating processes: sonicated the ITO electrode in an alconox solution (10g/L of alconox of double-distilled water) for 15 min, propan-2-ol for 15 min, and twice in double-distilled water for 15 min in sequence.[28]

RecJf Exonuclease-aided OTA Detection. The hybridization between probe DNA (ultimate concentration of 1.0 μM) and aptamer (ultimate concentration of 1.5 μM) occurs in 50 μL of 1×NEBuffer 2 at 37 °C for 2 h. After the hybridization process, $RecJ_f$ exonuclease (ultimate concentration of 0.04 unit/μL) and different concentrations of OTA were added in the above mentioned solution, incubated at 37 °C for 1.5 h to ensure the complete reaction. For the specificity test, OTB (7.0 ng mL^{-1}), OTC (7.0 ng mL^{-1}), or AFB1 (7.0 ng mL^{-1}) had been used to replace OTA. The DPV was carried out in 1×NEBuffer 2 in the potential range of 0 to -0.6 V. Each sample had been detected five times and the average value had been applied for quantitative analysis.

Fluorescence Measurement. The enzymatic reaction product was mixed with SYBR Green I (ultimate concentration of 0.8×) and then incubated at room temperature for 15 min. The fluorescence measurements were performed at room temperature with a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, USA). The emission spectra were collected from 500 to 600 nm with the excitation wavelength of 488 nm. Both the excitation and emission slit widths were set as 10.0 nm. The fluorescence intensity at 525 nm is used for analysis. Control group was conducted without the addition of OTA.

RESULTS AND DICUSSIONS

Principle of the Homogeneous Electrochemical aptasensor. The principle of a proposed homogeneous electrochemical aptasensor based on exonuclease-catalyzed target recycling is shown in Figure 1. The methylene blue (MB) labeled probe DNA had hybridized with the OTA aptamer first to form the double strand DNA (dsDNA) (step a). In the absence of the OTA，negligible electrochemical response can be detected due to the strong electrostatic repulsion between the dsDNA and the negative ITO electrode. At present of target OTA, the formation of aptamer-OTA complex will cause the dissociation of probe DNA from the dsDNA (step b). Then the probe DNA will be digested into mononucleotides including a MB-labeled electroactive mononucleotide (eT) due to the employment of the $RecJ_f$ exonuclease, which is a ssDNA specific exonuclease (step c). The eT can diffuse easily to the negative charged ITO electrode surface since the electrostatic repulsion from the negatively charged ITO working electrode to eT (which contains only 1-base) is much smaller than that from probe DNA or dsDNA. So a remarkable enhanced electrochemical response can be detected. At the same time, the aptamer in the aptamer-OTA complex can also be digested by the $RecJ_f$ exonuclease to liberate the OTA from OTA/aptamer and trigger the next round of complexion, dissociation and digestion. In this way, a single OTA can trigger signal production for many times and realize the signal amplification.

A simple experiment had been performed to verify our presumption. As shown in Figure 2A, in the absence of the OTA, the DPV signal was negligible (curve a). The

reason lies in that there has abundant negative charged on the dsDNA or the unhybridized probe DNA. At present of OTA, a remarkable enhanced electrochemical response was observed (curve b). Which can be attributed to large amount of negative charged eT's have been produced after interaction with OTA, which can diffuse easily to the negative charged ITO electrode surface and causes the enhancement DPV signal detected. These results clearly demonstrated the feasibility of our homogeneous electrochemical aptasensor. A fluorescence measurement was performed to further prove the feasibility of the proposed strategy. As shown in Figure 2B, a powerful fluorescence signal was acquired in the control group (curve a) dues to the abundant dsDNA polymers exist. After the addition of OTA, the fluorescence intensity decreased (curve b). This phenomenon verifies that the addition of OTA can cause complete double helices to come apart. The fluorescence signal decreased further after the addition of OTA and $RecJ_f$ exonuclease (curve c), that is due to the ssDNA released from complete double helices by OTA can be digested by $RecJ_f$ exonuclease and the OTA has been reused to cleave the dsDNA. This verified the amplification ability of the proposed method.

Optimization of the Reaction Conditions. The initial concentration of MB-labeled probe DNA is a key factor that affects the analytical properties of the biosensor. An excessive amount of probe DNA can result in the high background signal, while lower probe DNA concentration will cause the low electrochemical response. So the effect of probe DNA concentration had been optimized first. The concentrations of the OTA, aptamer and $RecJ_f$ exonuclease were set at 1.0 ng mL^{-1}, 1.5μM and 0.04

unit/μL respectively, and the concentrations of probe DNA were changed from 0.2 to 2.0 μM. The results showed that the DPV signal of the system increased with the enhancement of the probe DNA concentration and then reached the saturated condition after 0.8 μM (see Figure 3A). And the background signal enhanced also if the concentration of probe DNA was greater than 1.0 μM. Therefore, 1.0 μM probe DNA has been chosen as the optimized condition.

The hybridization time between the aptamer and probe DNA also plays an important role in the performance of the biosensor. Short time will result in the high background signal since large amount free probe DNA presents in the solution, which can be digested by the $RecJ_f$ exonuclease and produces high background electrochemical signal. As shown in Figure 3B, the ΔI (the difference of the currents detected at present and absent of OTA) increased with the increasing of hybridization time and reached a plateau after 2.0 h. Therefore, 2.0 h had been chosen as the optimized condition for the following study.

The effects of the target reaction time and the incubation temperature of $RecJ_f$ exonuclease on the signal detected were investigated too. The target reaction time was closely related to the formation of aptamer-OTA complex and the RecJf exonuclease-catalyzed OTA recycle. As shown in Figure 3C, the DPV signal increased with the increment of target reaction time from 30 to 90 min and then became stable. Therefore, 90 min had been applied in this experiment. Figure 3D showed that the DPV signal increased with an increment of incubation temperature up to 37 °C. However, the DPV signal decreased if the temperature changed to 43 °C.

The reason lies in that the $RecJ_f$ exonuclease's activity was low when the incubation temperature below the optimal temperature of the enzyme (37 °C). And high temperature will cause the inactive of the enzyme. So, 37 °C was selected as the optimized condition in this study.

Calibration Curve and Reproducibility of the Aptasensor. The sensitivity and dynamic range of the as-prepared aptasensor was evaluated under the optimized reaction conditions. The results showed that DPV signal increased gradually with the increasing of OTA concentration (shown in Figure 4A). Moreover, the enhanced DPV signal displayed a good linear relationship with OTA concentration in the range from 0.01 ng mL^{-1} to 1.0 ng mL^{-1} (see Figure 4 B). The regression equation is:

ΔI /nA = − 31.6514 − 76.4783Cx/ng mL^{-1}, R = 0.9937

where ΔI (the difference of the currents detected at present and absent of OTA) is enhancement of the DPV current, Cx is OTA concentration, and R is the regression coefficient. The limit of detection was estimated to be 0.004 ng mL^{-1} (S/N=3). Compared with the early reported aptasensors for OTA,[29-30] the proposed OTA biosensor apparently showed better or comparable sensitivity due to high efficiency of the $RecJ_f$ exonuclease-catalyzed target recycling amplification.

To examine reproducibility of the biosensor, one ITO working electrode was repeatedly used to detect 5 samples (0.1 ng mL^{-1}), the relative standard deviation (RSD) is 4.27%. And if 5 different ITO working electrodes are used for paralleling determination，the RSD is 5.03% . These results indicate that the proposed method has good repeatability.

Interference Study. The specificity of the proposed aptasensor had been investigated by replacing OTA with different molecules, such as OTB, OTC and AFB1. As shown in Figure 5, the DPV signal is negligible despite of the addition of interfering reagent at a concentration of 7.0 ng mL^{-1}. However, an obvious response with respect to 0.07 ng mL^{-1} OTA had been observed. The reason lies in the high specificity between OTA aptamer and OTA.

Application of the Aptasensor in Real Samples. To demonstrate its complete applicability, the proposed aptasensor has been applied to detect OTA in corn sample. The samples were extracted by 80% methanol solution firstly.[31] As shown in table 1, the detected values seem fairly similar with the reference concentrations. The standard addition recoveries had been performed to further verify the validity of the proposed aptasensor. It was found that recoveries were in the range of 93.8–97.6%. The proposed sensor also has been applied to detect OTA in the fresh and moldy oat samples. It was found no OTA in the fresh oat samples but about 300 ng g^{-1} in the moldy oat samples, and the recovery was in the range 94.5–101.0%. These results indicate that the proposed aptasensor had favourable reliability and can be applied to test the real food samples with satisfied results.

CONCLUSION

We presented a proof of concept for an exonuclease-catalyzed target recycling based immobilization free electrochemical aptasensor. The proposed aptasensor takes

advantage of the signal amplification of $RecJ_f$ exonuclease-catalyzed target recycling, the differential electrostatic repulsion between long and short DNA on a negatively charged ITO electrode surface and high selectivity of aptamer. Compared with most existing electrochemical aptasensor, the obvious advantage of as-prepared aptasensor was high reaction efficiency and easy operation without the tedious probe immobilization processes. What's more, the proposed biosensor can be applied to the quantitative determination of OTA from the real samples.

ACKNOWLEDGEMENTS

This work was financially supported NSFC for Excellent Youth Scholars of China (21222506), NSFC (21175024, 21275031), Nature Sciences Funding of Fujian Province(2014J06005), and program for New Century Excellent Talents in University (NCET-12-0619).

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Figures and Captions

Figure 1. Scheme of the immobilization free electrochemical aptasensor for OTA.

Figure 2. (A) Feasibility of the electrochemical biosensor: (a) 0 ng mL^{-1} OTA; (b) 1.0 ng mL^{-1} OTA; (B) Fluorescence measurement: (a) 0 ng mL^{-1} OTA; (b) 1.0 ng mL^{-1} OTA+ $RecJ_f$ exonuclease; (c) 1.0 ng mL^{-1} OTA. [Probe DNA] = 1 μM, [Aptamer] = 1.5 μM, [$RecJ_f$ exonuclease] = 0.04 unit/μL, [SYBR Green I] = 0.8×. The figures on the right are the corresponding schematic illustrations.

Figure 3. (A) Effect of the probe DNA concentration on the DPV response. (B) Effect of the dsDNA self-assembly time on the enhancement of the DPV current. (C) Effect of the target reaction time on the DPV response. (D) Effect of incubation temperature of $RecJ_f$ exonuclease on the DPV response. [Probe DNA] = 1 μM, [Aptamer] = 1.5 μM, [$RecJ_f$ exonuclease] = 0.04 unit/μL, [OTA] = 1.0 ng mL^{-1}.

Figure 4. (A) DPV responses at different OTA concentration. (a) 0 ng mL^{-1}, (b) 0.01 ng mL^{-1}, (c) 0.1 ng mL^{-1}, (d) 0.3 ng mL^{-1}, (e) 0.5 ng mL^{-1}, (f) 0.7 ng mL^{-1}, (g) 1.0 ng mL^{-1}. (B) The relationship between the enhancement of DPV current and the OTA concentration. [Probe DNA] = 1μM, [Aptamer] = 1.5μM, [$RecJ_f$ exonuclease] = 0.04 unit/μL.

Figure 5. Specificity of the electrochemical biosensor: (a) 0.7 ng mL^{-1} OTA, (b) 7.0 ng mL^{-1} OTB, (c) 7.0 ng mL^{-1} OTC, (d) 7.0 ng mL^{-1} AFB1, and (e) Control. [Probe DNA] = 1μM, [Aptamer] = 1.5μM, [$RecJ_f$ exonuclease] = 0.04 unit/μL.

Figure 1

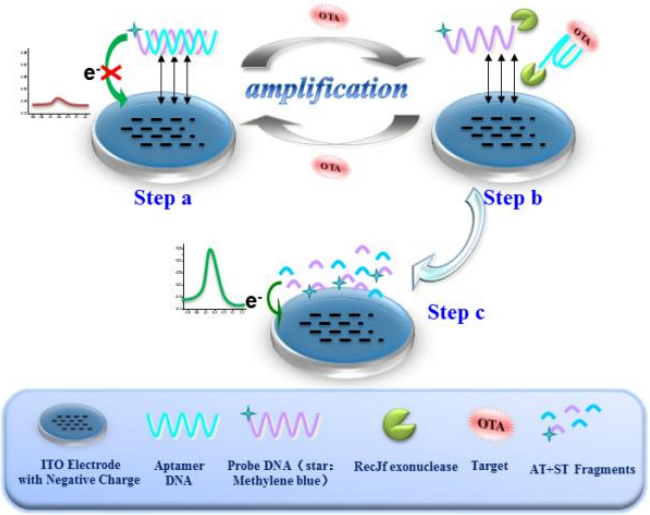
OTA
amplification
OTA
OTA
e-
Step a
Step b
e-
Step c
ITO Electrode with Negative Charge
Aptamer DNA
Probe DNA（star: Methylene blue）
RecJf exonuclease
OTA
Target
AT+ST Fragments

Figure 2

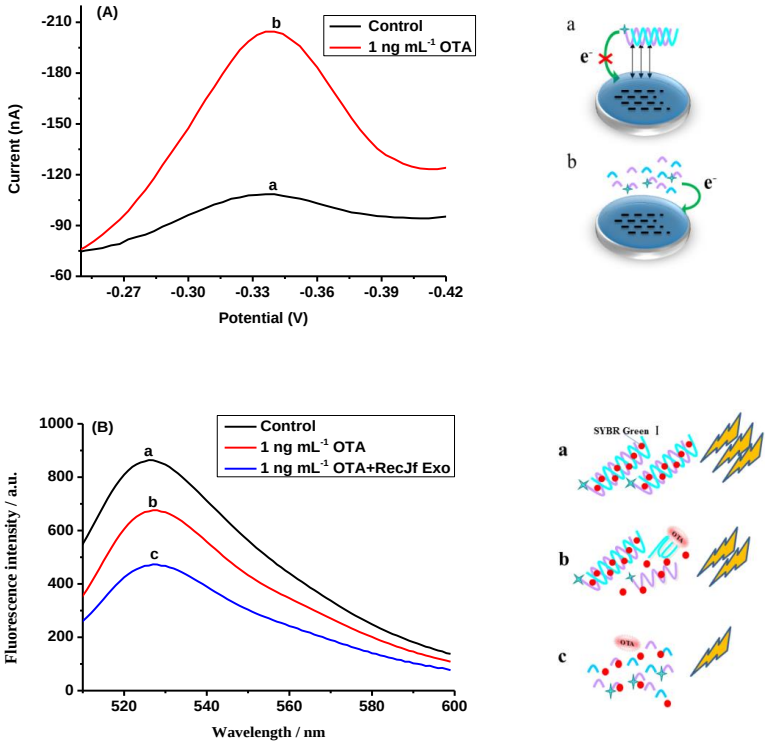
(A)
Control
1 ng mL-1 OTA
Current (nA)
Potential (V)
a
b
e-
(B)
Control
1 ng mL-1 OTA
1 ng mL-1 OTA+RecJf Exo
Fluorescence intensity / a.u.
Wavelength / nm
SYBR Green I
OTA

Figure 3

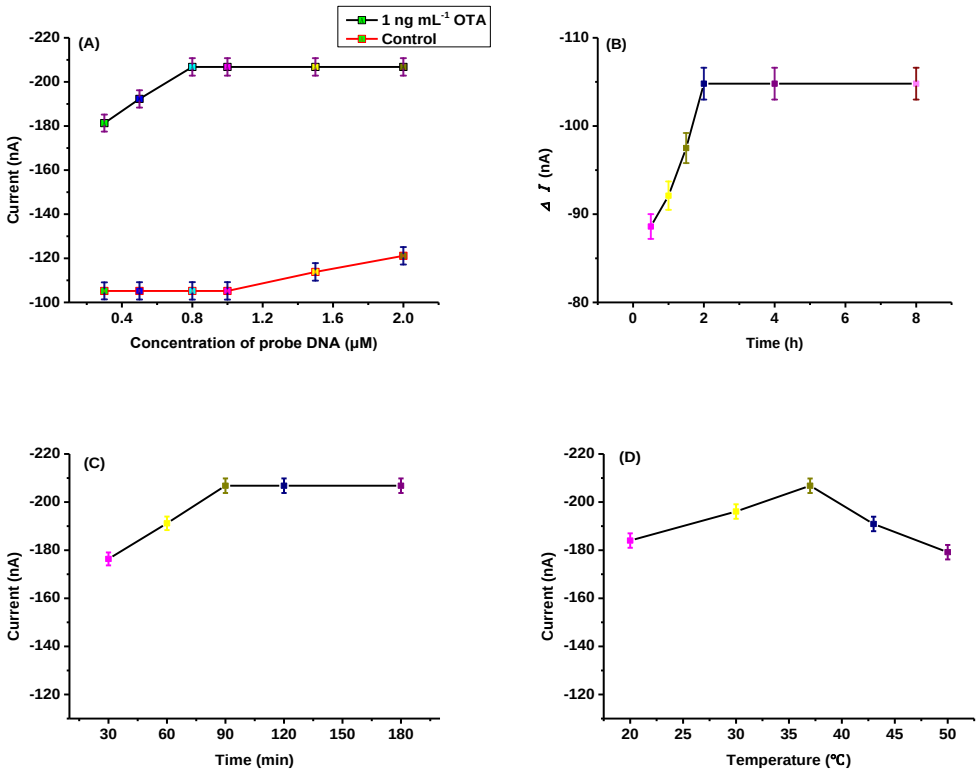
(A)
1 ng mL-1 OTA
Control
Current (nA)
Concentration of probe DNA (μM)
(B)
Δ I (nA)
Time (h)
(C)
Current (nA)
Time (min)
(D)
Current (nA)
Temperature (℃)

Figure 4

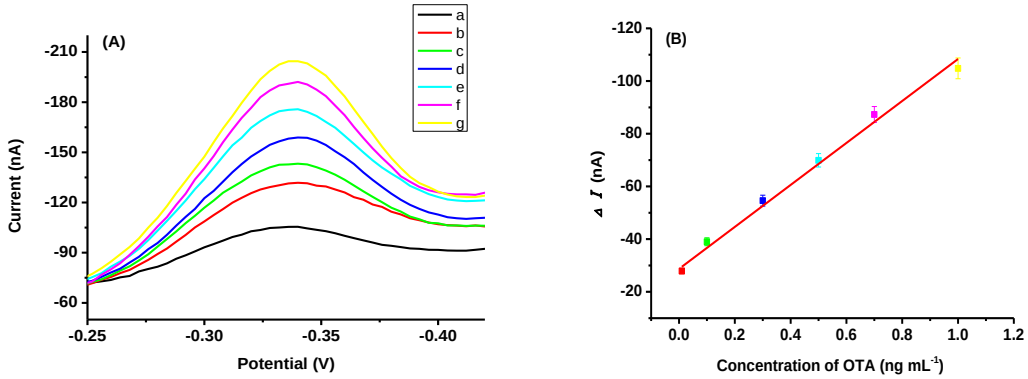
(A)
Current (nA)
Potential (V)
-210
-180
-150
-120
-90
-60
-0.25
-0.30
-0.35
-0.40
a
b
c
d
e
f
g
(B)
Δ I (nA)
Concentration of OTA (ng mL-1)
-120
-100
-80
-60
-40
-20
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure 5

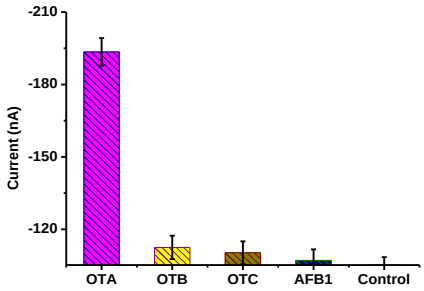

Table 1 Determination of OTA in corn samples and oat samples[a]（n=5）

Sample		Detected (ng g $^{-1}$)	Reference (ng g $^{-1}$)	Spiked (ng g $^{-1}$)	Total found (ng g $^{-1}$)	Recovery (%)	RSD (%)
Corn	1	47.9	54.2	10.0	57.4	95.0	4.9
	2	48.6	54.2	20.0	67.9	96.5	5.1
	3	49.1	54.2	40.0	88.2	97.8	5.6
Fresh oat	1	-	/	10	10.3	103.0	5.2
	2	-	/	40	39.2	98.0	5.4
Moldy oat	1	345	/	10	355.1	101.0	5.1
	2	319	/	40	356.8	94.5	5.3

[a] **"/" means unknown; "-"means lower the detection limits of the proposed method.**

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