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Electrochemiluminescence recovery-based aptasensor for sensitive Ochratoxin A detection via exonuclease-catalyzed target recycling amplification

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ABSTRACT

Based on the recovery of the quantum dot (QD) electrochemiluminescence (ECL) and exonuclease-catalyzed target recycling amplification, the development of a highly sensitive aptasensor for Ochratoxin A (OTA) detection is described. The duplex DNA probes containing the biotin-modified aptamer are immobilized on a CdTe QD composite film-coated electrode. The presence of the OTA target leads to effective removal of the biotin–aptamers from the electrode surface via exonuclease-catalyzed recycling and reuse of OTA, which prevents the attachment of streptavidin–alkaline phosphatase (STV–ALP) through biotin–STV interaction. The electron transfer (ET) from the excited state CdTe QD ([CdTe]*) to the electro-oxidized species of the enzymatic product of ALP during the potential scan is thus inhibited and the QD ECL emission is restored for quantitative OTA detection. Due to the exonuclease-catalyzed target recycling amplification, the inhibition effect of ET is significantly enhanced to achieve sensitive detection of OTA down to 0.64 pg mL^{-1}. The proposed method is selective for OTA and can be used to monitor OTA in real red wine samples. Our developed ECL recovery-based aptasensor thus offers great potential for the development of new ECL sensing platforms for various target analytes.

1. Introduction

Ochratoxin A (OTA), N-[(3R)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-iso-chromanyl) carbonyl]-L-phenylalanine, a mycotoxin produced by certain strains of filamentous fungi of *Penicillium* and *Aspergillus*, is reported to be one of the most toxic and cancerigenic substances to a wide variety of mammalian species [1,2]. It has been reported that OTA exists in various food commodities, including cereals, wheat, beans, nuts, coffee and wine [3–6]. Toxicology studies also have shown that OTA has nephrotoxicity, hepatic and immune toxicity, and has carcinogenic effects on humans [7]. Therefore, the identification and detection of OTA is highly important to prevent food contamination related to human health.

Traditional methods reported for OTA detection mainly relied on the chromatographic approaches [8,9], including high performance liquid chromatography and thin layer chromatography. The high cost, sophisticated equipments and the need for professional skills have limited the practical application of these methods. Despite the immunoassays based on antigen–antibody interactions such as enzyme-linked immunosorbant assays (ELISAs) have shown improvements over the traditional methods, the production of antibodies against toxins and achieving high sensitivity remain challenges. The development of new alternatives for OTA determination is thus highly demanded.

Aptamers are artificial nucleic acids, which can bind with target molecules (e.g., proteins, cells, amino acids, organic and inorganic ions) with high affinity and specificity [10,11]. Compared with antibodies, aptamers exhibit significant advantages such as low cost, target versatility, thermal stability, easy synthesis and modification. One of the attractive features of aptamers is that these artificial nucleic acid probes can specifically bind with toxic or highly toxic targets and can be in vitro selected [12–14]. Since the first report on the DNA aptamer selectively bound to OTA by Cruz-Aguado and Penner [15], several aptamer-based biosensors (aptasensor) for OTA based on fluorescence [16,17], colorimetry [18] and electrochemistry [19–21] have been reported. Among these detection schemes, electrochemical analysis of OTA appears to be one of the effective approaches because of the low cost, simplicity and high sensitivity of this technique. Electrochemiluminescence (ECL) is a chemiluminescent reaction triggered by electrochemical methods [22]. Combined with the advantages of chemiluminescence and

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electrochemistry, ECL biosensors have been widely developed owing to their simplicity, high sensitivity and potential controllability. In recent years, quantum dots (QDs) have been increasingly used to construct different ECL biosensors due to the chemical stability, efficient and stable ECL signals of the QDs [23,24], and special attentions have been given to ECL quenching-based bioassays. The QD ECL emission can be quenched by quenchers such as quinones [25], gold nanoparticles [26,27] and gold nanorods [28], which has extended the application of ECL in bioanalyses.

Herein, we report on a new ECL aptasensor platform for sensitive detection of OTA based on ECL recovery and exonuclease-catalyzed target recycling amplification. During the potential scan, the ET takes place between the [CdTe]* and the electro-oxidized species of the enzymatic product of ALP. The ET is inhibited with the presence of OTA and the ECL emission of the CdTe QDs is restored. The restoration of the ECL emission is dramatically amplified due to the exonuclease-catalyzed recycling and reuse of OTA. The exonuclease-catalyzed recycling of the target molecules have been shown to be an effective signal amplification route to achieve high sensitivity with a new one to N instead of the traditional one to one target/probe recognition ratio [29–31]. By using this new and powerful exonuclease-catalyzed target recycling signal amplification approach, we show below that highly sensitive OTA detection can be realized by using aptamer probes.

2. Experimental

2.1. Materials and reagents

OTA and ochratoxin B (OTB) were purchased from Shanghai BioSun Sci & Tech CO., LTD (Shanghai, China). $RecJ_f$ exonuclease with 1 × NEB buffer 2 was obtained from New England Biolabs (Beijing, China). N-(3-dimethylamminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimid sodium salt (NHS), triethylamine (TEA), thrombin, 3-mercaptopropionic (MPA), cocaine and chitosan (CS) were bought from Sigma (St. Louis, MO). $NaBH_4$, $CdCl_2$ and trisodium citrate dihydrate were obtained from Kelong Chemical Company (Chengdu, China). Na_2TeO_3 was received from J&K Scientific Ltd. (Guangzhou, China). Multi-wall carboxyl carbon nanotubes (MWCNT) (> 95% purity) and *p*-aminophenylphosphate (*p*-APP) were supplied by Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China) and LKT Laboratories Inc. (St Paul, MN, USA), respectively. The streptavidin-conjugated alkaline phosphatase (STV–ALP) was purchased from Promega (USA). The oligonucleotides with the following sequences were synthesized and purified by Shanghai Sangon Biotechnology (Shanghai, China). OTA aptamer [21]: 5'-AAA GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA–biotin-3'. Complementary DNA (C-DNA): 5'-CCG ATG CTC CCT TTA CGC CAC CCA CAC CCG ATC CC-$(CH_2)_6$–NH_2-3'. The OTA stock solution (1 mg mL^{-1}) was prepared by dissolving OTA (1.0 mg) in ethanol (10.0 mL). The OTA working solutions were freshly prepared by serial dilution of the stock solution in water. All reagents were of analytical grade and solutions were prepared using ultrapure water (specific resistance of 18 MΩ-cm).

2.2. Apparatus

The ECL emission was recorded on a MPI-A ECL analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., Xi'an, China) in the ECL detection buffer (50 mM Gly, 0.5 mM *p*-APP, 0.5 mM $MgCl_2$ and 10 mM TEA, pH 9.5) with the modified glassy carbon electrode (Ø = 3 mm) as the working electrode, a platinum wire as the counter electrode and a Ag/AgCl as the reference electrode. The working potential was 0–1.6 V with the voltage of the photomultiplier tube set at 800 V and the scan rate set at 100 mV s^{-1}. The electrochemical impedance spectroscopy (EIS) measurements were performed on a CHI 660D EC workstation (CH Instruments Inc., Shanghai, China) in the 0.1 M KCl solution containing 5 mM (1:1) $[Fe(CN)_6]^{3-/4-}$. EIS measurements were recorded within the range from 100 kHz to 0.1 Hz and an alternate voltage of 5 mV.

2.3. Preparation of the water soluble MPA-capped CdTe QDs

The synthesis of the CdTe QDs was performed according to previously reported methods [32,33]. Briefly, $CdCl_2$ (36.54 mg) powder was dissolved in pure water (49 mL), followed by the addition of trisodium citrate dihydrate (50 mg), Na_2TeO_3 (0.01 M, 1 mL), MPA (33 μL) and $NaBH_4$ (50 mg) with stirring. Next, a three-necked flask containing the above mixture was connected to a condenser and refluxed at 110 °C for 10 h. The obtained CdTe QDs were then washed with ethanol twice, separated by centrifugation, dispersed in water, and stored at 4 °C for further use.

2.4. Preparation of the NH_2-C-DNA/aptamer–biotin duplex probes

The OTA aptamer were hybridized with the amine-modified C-DNA to form the duplex probes by mixing 10 μL of 2 μM C-DNA and 10 μL of 2.4 μM aptamer in 0.1 M PBS annealing buffer (pH 7.4). The resulting mixture was then heated to 90 °C for 5 min and kept at 65 °C for 10 min, followed by gradually cooling down to room temperature. The duplex probes were stored at 4 °C for further use.

2.5. ECL recovery-based OTA sensing protocol

Prior to modification, the glassy carbon electrodes (GCEs) were polished to mirror-like surfaces using 0.3 and 0.05 μm alumina slurry, followed by sequential sonication in ethanol and water for 5 min. After being dried in N_2 atmosphere, 10 μL of CdTe–MWCNT–CS (mixture of 0.30 mg mL^{-1} CS/MWCNT and 0.05 mM CdTe) solution was covered on the electrode surface and dried in a refrigerator overnight. (Note: MWCNTs possess excellent electrical conductivity and porous structure, which can compensate the poor conductivity of the CdTe QDs and facilitate the ECL reaction for sensitive ECL detection [34]). Next, the electrodes were incubated with a freshly prepared mixture of EDC (400 mM) and NHS (100 mM) for 30 min to activate the carboxyl groups on the surfaces. After rinsing with water, 10 μL of the NH_2-C-DNA/aptamer–biotin duplex probes (1 μM) was dropped onto the electrode surface for 2 h (the electrodes were covered with a plastic cap to avoid the solution evaporation). Afterwards, the resulting electrodes were carefully washed with 0.1 M PBS (pH 7.4) to remove the unbound probes and blocked with 1 mM ethanolamine for 30 min at room temperature. The modified electrodes were then incubated with 20 μL of various concentrations of OTA in 1 × NEB buffer 2 containing 4.5 U $RecJ_f$ exonuclease for 90 min at 37 °C, followed by washing with 0.1 M PBS. Subsequently, 10 μL of STV–ALP (1 μg mL^{-1}) was dropped on the electrodes for 30 min and rinsed with 0.1 M PBS. Finally, the sensors were transferred into the ECL detection buffer and incubated for 5 min, followed by ECL measurements.

3. Results and discussion

3.1. Principle of the target recycling amplification and ECL recovery-based aptasensor for OTA detection

The concept of our exonuclease-catalyzed ECL recovery for sensitive detection of OTA is depicted in Scheme 1. The sensing

surface is constructed by the deposition of a CdTe–MWCNT–CS composite film on the GCE surface, followed by covalent coupling of the NH_2-C-DNA/aptamer–biotin duplex probes on the surface. In the absence of the OTA target, the $RecJ_f$ exonuclease, which shows specific activity for catalytic removal of deoxynucleotide monophosphates toward ssDNA in the 5' to 3' direction, is inactive to the surface immobilized duplex probes. The biotin moieties of the probes can thus capture the STV–ALP on the electrode surface through strong biotin–STV binding. The surface-captured ALP subsequently catalyzes the conversion of the *p*-APP substrate to *p*-aminophenol (*p*-AP) in the detection buffer, which is electro-oxidized to *p*-quinone imine (*p*-QI) during the potential sweep. At the same time, the excited CdTe quantum dots ([CdTe]*) are also formed during the potential sweep with the presence of the co-reactant (TEA). The collisions between the [CdTe]* and the electro-oxidized *p*-QI result in ET from the [CdTe]* to *p*-QI [35], leading to significant quenching of the ECL emission intensity of the CdTe QDs. On the contrary, when the OTA target is present, it associates with and releases the biotin-modified aptamer of the duplex probes from the electrode surface. The OTA associated, biotin-modified aptamer can thus be digested by the $RecJ_f$ exonuclease to release the OTA target. The released OTA again associates with the intact biotin-modified aptamer on the electrode surface to initiate the OTA recycling process, which results in the removal of a large number of biotin-modified aptamer from the electrode surface and the capture of less ALP. The production of *p*-AP and subsequent electro-oxidized *p*-QI is thus minimized, leading to inhibited ET from the [CdTe]* to *p*-QI and restoration of the ECL emission of the CdTe QDs. Due to the recycling and reuse of the OTA target, the restoration of the ECL emission intensity of the CdTe QDs is expected to be significantly amplified to achieve sensitive detection of OTA.

3.2. EIS characterization of the modified electrode surfaces

The stepwise modifications of the sensing electrode surface were monitored by EIS. In a typical EIS spectrum, the diameter of the semicircle reveals the electron transfer resistance (R_{et}). The value of R_{et} varies when different materials are absorbed onto the electrode surface. As shown in Fig. 1, the bare GCE exhibits very small R_{et} (curve a) due to the excellent conductivity of the bare GCE. After the GCE is coated by the CdTe–MWCNT–CS composite film, the R_{et} (curve b) is apparently increased mainly because the deposition of the CdTe QDs and chitosan increases the impedance. The covalent attachment of the NH_2-C-DNA/aptamer–biotin duplex probes and subsequent surface blocking by ethanolamine result in further increases of R_{et} (curve c and d) owing to the steric hindrance from the rigid duplex structures and the insulating properties that resist the electron transfer kinetics of the redox probe at the electrode interface. However, significant decrease in R_{et} can be observed after the target OTA (5.0 ng mL^{-1}) and $RecJ_f$ exonuclease (4.5 U) are introduced to the sensing surface (curve e), originating from the effective removal of the aptamers as discussed previously and reduced steric hindrance of the electrode surface. These EIS results sufficiently indicate the successful construction of the aptasensor.

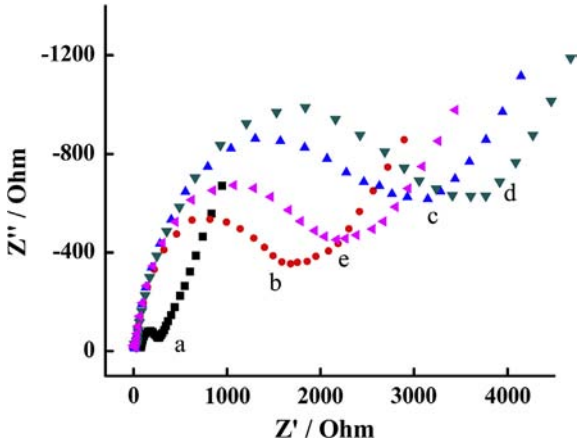
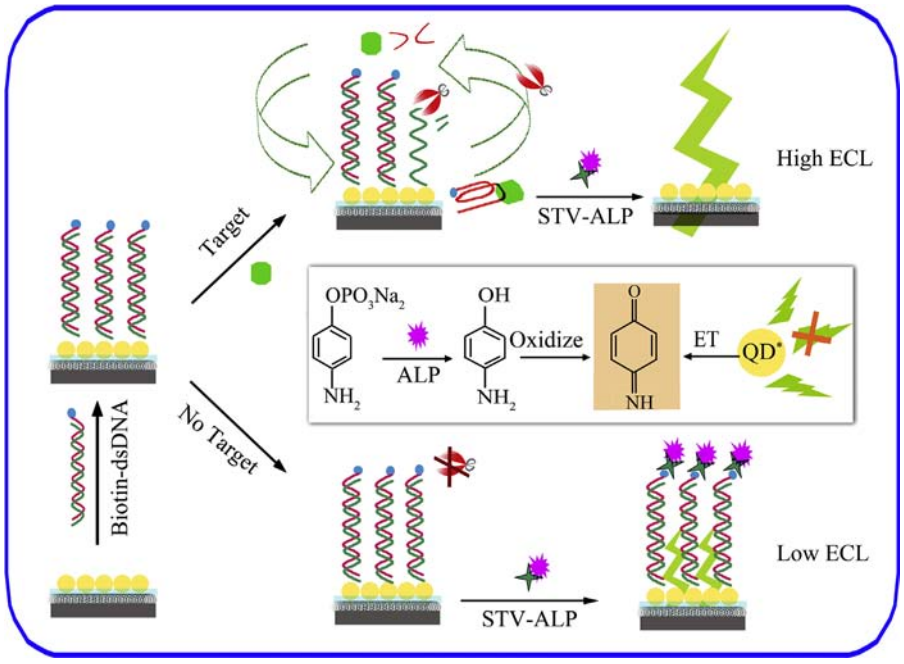


Fig. 1. EIS plot of the stepwise modification of the electrode: (a) bare GCE, (b) CdTe–MWCNT–CS/GCE, (c) dsDNA/CdTe–MWCNT–CS/GCE, (d) ethanolamine/dsDNA/CdTe–MWCNT–CS/GCE, and (e) OTA-$ReCJ_f$/ethanolamine/dsDNA/CdTe–MWCNT–CS/GCE. The EIS measurements were recorded in 0.1 M KCl solution containing 5 mM (1:1) $[Fe(CN)_6]^{3-/4-}$ as the redox probes within the range from 100 kHz to 0.1 Hz at the formal potential of $[Fe(CN)_6]^{3-/4-}$.



Scheme 1. Illustration of the principle of the exonuclease-catalyzed target recycling amplification for sensitive OTA detection based on the recovery of the QD ECL.

3.3. CdTe QD ECL recovery mechanism

Toward the validation of the proposed protocol, the ET quenching of the CdTe ECL emission by electro-generated *p*-QI was first investigated. During the potential scan, as the potential of the sensing electrode becomes sufficiently positive, the CdTe QDs coated on the electrode are electro-oxidized to [CdTe] $(h)^+$ by a hole injection and TEA is oxidized to $TEA^{\bullet}$ radicals. The hole-injected [CdTe] $(h)^+$ radicals collide with $TEA^{\bullet}$ to produce the excited state [CdTe]*, which can emit light and produce an ECL signal when return to the ground state. The corresponding ECL mechanism can be expressed as follows:[36]

$$[CdTe] - e^- \rightarrow [CdTe](h)^+ \quad (1)$$

$$TEA - e^- \rightarrow [TEA^{\bullet}]^+ \rightarrow TEA^{\bullet} + H^+ \quad (2)$$

$$[CdTe](h)^+ + TEA^{\bullet} \rightarrow [CdTe]^* + \text{TEA fragments} \quad (3)$$

$$[CdTe]^* \rightarrow [CdTe] + h\upsilon \quad (4)$$

By following the mechanism of the CdTe ECL emission, a strong ECL response is observed on the ethanolamine/dsDNA/CdTe–MWCNT–CS/GCE sensing electrode (curve a in the inset of Fig. 2). However, after the incubation of the sensing electrode with $RecJ_f$ exonuclease in the absence of OTA and subsequent addition of STV–ALP, the ECL emission of the CdTe QDs is significantly quenched (curve b in Fig. 2 and the inset) due to the ET from the [CdTe]* to *p*-QI, resulting from electro-oxidation of *p*-AP generated by the surface-captured ALP [25,37].

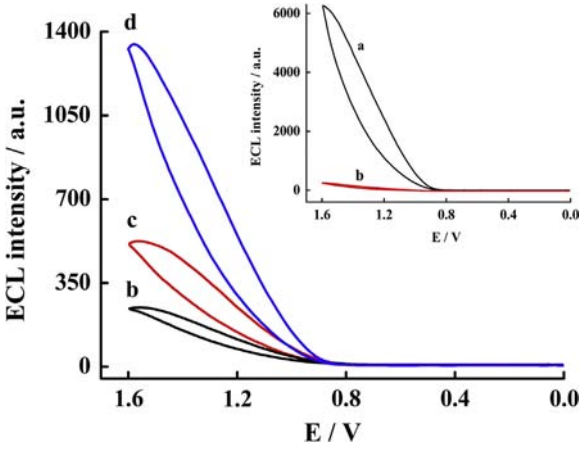


Fig. 2. ECL intensity of the aptasensor in the presence of (b) $RecJ_f$ exonuclease (4.5 U), (c) OTA (5.0 ng mL^{-1}) and (d) $RecJ_f$ exonuclease (4.5 U) and OTA (5.0 ng mL^{-1}) following by incubation with STV–ALP (1.0 μg mL^{-1}). Inset: ECL intensity of the aptasensor incubated (a) without and (b) with STV–ALP (1.0 μg mL^{-1}) in the absence of OTA. ECL measurements were carried out in ECL detection buffer containing 50 mM alkaline Gly, 0.5 mM *p*-APP, 0.5 mM $MgCl_2$ and 10 mM TEA. The voltage of the photomultiplier tube: 800 V; scan rate: 100 mV s^{-1}.

For proof-of-concept demonstration of the amplified ECL recovery-based approach for sensitive detection of OTA, the sensing electrode is incubated with a solution of OTA (5 ng mL^{-1}) in the absence of the $RecJ_f$ exonuclease for 60 min, followed by the addition of STV–ALP. From Fig. 2, we can see that the presence of OTA (without $RecJ_f$) leads to partial restoration of the ECL emission of the CdTe QDs (curve c) compared with the blank test (0 ng mL^{-1} of OTA and 4.5 U $RecJ_f$, curve b). Such ECL restoration mainly derives from OTA-induced release of the corresponding biotin-modified aptamers from the sensing electrode, which decreases the capture of ALP and thus inhibits ET quenching of the CdTe QD ECL emission. Importantly, when OTA (5 ng mL^{-1}) and $RecJ_f$ exonuclease (4.5 U) simultaneously exist in the incubation solution, a dramatically amplified restoration of ECL emission is observed (curve d vs c in Fig. 2), which is due to the catalytic target recycling by $RecJ_f$ exonuclease and effective removal of the biotin-modified aptamers from the sensing electrode for preventing the attachment of ALP. These results shown here clearly demonstrate the ET quenching of the QD ECL emission by electro-generated *p*-QI and significantly amplified inhibition of the quenching effect by $RecJ_f$ exonuclease-catalyzed OTA target recycling.

3.4. Optimization of the assay conditions

The ECL responses of the aptasensor for OTA detection are dependent on the assay conditions such as the amount of $RecJ_f$ exonuclease and enzymatic cleavage time. Fig. 3A shows the effect of the amount of $RecJ_f$ (in the range from 3.0 U to 5.5 U) on the ECL intensity of the sensor. It can be seen that the ECL intensity increases with increasing amount of $RecJ_f$ and reaches maximum at 4.5 U. The effect of the enzymatic cleavage time was also investigated from 45 to 120 min with 15 min interval. As shown in Fig. 3B, it is apparent that the ECL intensity increases along with prolonged incubation time and levels off after 90 min, which means that 90 min is enough for the OTA binding and exonuclease cleavage.

3.5. Calibration curve and stability of the sensor

Under optimal conditions, quantitative detection of OTA using the proposed ECL recovery aptasensor was demonstrated by incubating the aptasensor with different concentrations of the target OTA. As shown in Fig. 4A, the addition of increasing

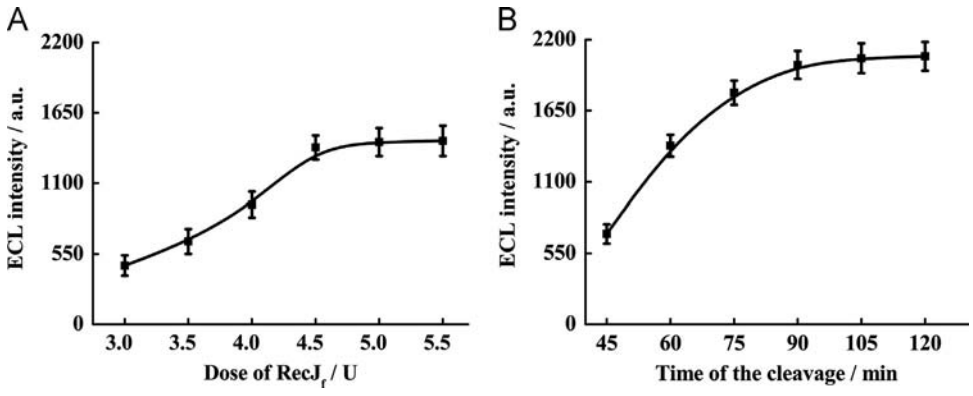


Fig. 3. Effects of (A) the amount of $RecJ_f$ exonuclease and (B) the enzymatic cleavage time on the ECL intensity of the aptasensor for OTA (5 ng mL^{-1}) detection. Other conditions, as in Fig. 2.

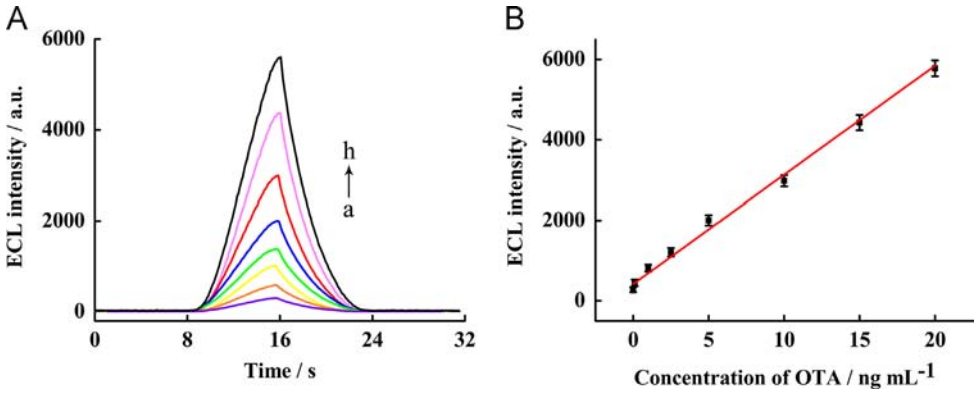


Fig. 4. (A) ECL intensity of the proposed aptasensor for the determination of different concentrations of OTA (a) 0.001, (b) 0.1, (c) 1, (d) 2.5, (e) 5, (f) 10, (g) 15 and (h) 20 ng mL^{-1}. (B) The corresponding calibration curve of (A). Error bars, SD, $n=3$. Other conditions, as in Fig. 2.

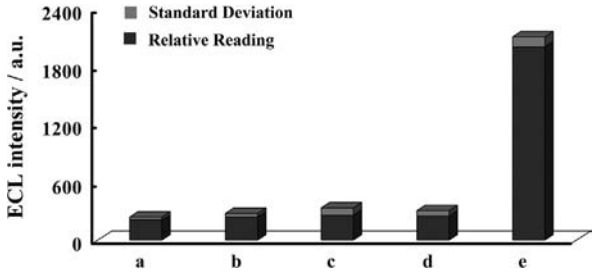


Fig. 5. Selectivity investigation of the aptasensor for the detection of OTA against other control molecules: (a) blank, (b) thrombin (50 ng mL^{-1}), (c) cocaine (50 ng mL^{-1}), (d) OTB (50 ng mL^{-1}), and (e) OTA (5 ng mL^{-1}).

concentration of OTA results in a dynamic increase in ECL signals, consistent with a lower amount of *p*-QI generated upon the decreased attachment of the STV–ALP. By plotting the ECL intensity versus the concentration of OTA, the calibration curve is obtained. As shown in Fig. 4B, the linear range for OTA detection with the proposed aptasensor is from 0.001 to 20 ng mL^{-1} ($R=0.997$). Based on the 3σ rule, the limit of detection (LOD) is calculated to be 0.64 pg mL^{-1}. Such LOD is much lower than the present acceptable safe OTA concentration of 3 ng mL^{-1} in cereals for human consumption and is more sensitive than other reported sensors based on colorimetric [18], fluorometric [38] and electrochemical [39] methods. We also performed the stability tests for the sensors by detecting OTA (5 ng mL^{-1}) with sensors from the same batch after 7 and 10 days of storage at 4 °C. We observed 5% (after 7 days) and 12% (after 10 days), decreases in signal the ECL output. These signal decreases might be due to the loss of the stability of the probes on the sensor surface.

3.6. Selectivity of the proposed aptasensor for the detection of OTA

The selectivity of the aptasensor is of great importance for the practical implementation of OTA detection. To evaluate the specificity of our OTA detection method, three other control molecules including thrombin, cocaine and OTB were also tested. As shown in Fig. 5, the ECL intensities of the control molecules (50 ng mL^{-1}) are close to that of the blank test while the presence of OTA with even at 10-fold less concentration (5 ng mL^{-1}) shows significant increase in ECL intensity compared to the control molecules, which reflects the high binding specificity of the OTA aptamer to the OTA target analyte. In other words, the developed aptasensor shows high selectivity for the OTA target against other interference molecules.

Table 1
Recovery of OTA from the red wine samples.

Samples	Amount of spiked OTA (ng mL^{-1})	Method and recovery (%)	
		Aptasensor	ELISA
1	0	0.45 ± 0.18 (−)	0.49 ± 0.20 (−)
2	0.1	0.56 ± 0.22 (110%)	0.60 ± 0.25 (110%)
3	0.5	0.93 ± 0.24 (96%)	0.96 ± 0.28 (94%)
4	1	1.53 ± 0.29 (108%)	1.61 ± 0.31 (112%)
5	5	5.65 ± 0.32 (104%)	5.74 ± 0.34 (105%)

3.7. Evaluation of OTA in red wine samples

In order to evaluate the practical applicability of the proposed method, the recoveries of five different concentrations of OTA with standard addition in real red wine samples were investigated and the results were compared with those obtained from an ELISA kit. As shown in Table 1, the recoveries of the developed aptasensor are in an acceptable range from 96% to 110% ($n=7$), and show negligible differences with those measured with the ELISA kit. These results indicate that the proposed ECL recovery-based aptasensor has the potential to be applied to monitor OTA in real samples.

4. Conclusion

In conclusion, we have shown a highly sensitive aptasensor for OTA detection based on the recovery of the QD ECL and exonclease-catalyzed target recycling amplification. The ET from the excited state CdTe QDs ([CdTe]*) to electro-oxidized product of *p*-AP leads to the quenching of QD ECL emission. With exonuclease-catalyzed OTA target recycling, this quenching effect is significantly inhibited and the ECL emission is restored in the presence of OTA. The proposed method exhibits a low detection limit of 0.64 pg mL^{-1} for OTA and is selective against other control molecules. The strategy can also be applied for the detection of OTA in red wine real samples. Compared with other methods, this method not only provides a new route to fabricate ECL sensors, but also indicates a promising screening platform for various analytes.

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