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ORIGINAL PAPER

A fluorescent aptasensor for ochratoxin A detection based on enzymatically generated copper nanoparticles with a polythymine scaffold

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Abstract

A fluorescence enhancement method is presented for the determination of ochratoxin A (OTA). The interaction of OTA with its aptamer causes structural changes which, in turn, change fluorescence of enzymatically generated polythymine-coated copper nanoparticles (CuNPs) (with excitation/emission maxima at 340/625 nm). The OTA-binding aptamer was immobilized on magnetic beads. When it binds OTA, it is partially released and exposes a region with a partly complimentary DNA strand (cDNA). After magnetic separation, the cDNA was employed as a primer to trigger the terminal deoxynucleotidyl transferase-mediated polymerization. This process generates polythymine which act as a template for synthesis of the CuNPs. The method is sensitive in having a 2.0 nM detection limit for OTA. It was successfully applied to the determination of OTA in spiked diluted red wine.

Keywords Aptamer · Biosensor · DNA · Mycotoxins · Metal nanoparticles · Enzymatic polymerization · Food safety · Terminal deoxynucleotidyl transferase · Structure switching · Red wine

Introduction

Ochratoxin A (OTA) is generated by *Aspergillus ochraceus, Penicillium verrucosum, and Penicillium nordicum* [1]. OTA contaminates a variety of foodstuffs, such as wine, beer, coffee beans, cereals, nuts and animal feed [2]. Toxicological studies have shown that OTA has strong nephrotoxicity, hepatotoxicity, immunotoxicity, mutagenicity, carcinogenicity, teratogenic toxicity and neurotoxicity [3]. OTA endangers human health seriously due to its long half-life, high chemical stability and thermal stability, and can be enriched in the human body through the food chain [4]. In view of its widespread distribution and perniciousness, OTA is classified as a potential carcinogen for humans (group 2B) by the International Agency for Research on Cancer (IARC) [5]. Thus, it is highly important to sensitively and precisely detect OTA in contaminated agricultural and food species to avoid OTA consumption.

Aptamers are single-stranded oligonucleotides (ssDNA or ssRNA sequences), which are artificially produced via in vitro selection process named systematic evolution of ligands by exponential enrichment (SELEX). They can bind to a variety of targets, including small molecules, proteins, cells, and even organisms with high specificity and affinity [6]. Various aptamer-based methods for the detection of OTA have been reported since its aptamer has been reported in 2008 [7]. Some of these have made some progress in their high sensitivities based on techniques, such as electrochemical [8], colorimetry [9–12], surface enhanced Raman scattering [13], lateral flow assay [14], fluorometry [15–20], and so on. For example, Wang et al. developed an amplified electrochemical aptasensor based on beta-cyclodextrin host-guest recognition for OTA detection [8]. Zhou et al. constructed a DNA hydrogel which

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encapsulates horseradish peroxidase for colorimetric detection of OTA [9]. A detection limit of 10.8 nM was attained. However, most of these techniques need deliberate modifications of electrode surfaces or sophisticated probes preparation, which is labour-intensive and costly. Therefore, a convenient synthesis material will be encouraging for OTA detection.

Rotaru et al. reported that double-stranded DNA (dsDNA) can be used as commendable templates for CuNP preparation [21]. The metallization can be quickly achieved with mild reducing agent ascorbic acid/ascorbate at room temperature. Besides, CuNPs exhibit prominent spectral properties (large Stokes shift and bright fluorescence), and low toxicity. Based on these prominent advantages, dsDNA-templated CuNPs have been wildly used as fluorescence indicators in the field of bioanalysis [18, 22–25]. Song et al. for the first time developed a sensitive fluorescent method for OTA assay based on dsDNA-templated CuNPs. By coupling with RecJf exonuclease catalyzed target recycling amplification innovatively, a low detection limit of 5 ng·mL^{-1} was attained [18].

Considering that dsDNA needs an extra hybridization step, ssDNA is a superior template for metal nanomaterials formation. Qing et al. [26] demonstrated that when poly(thymine) ssDNA was used as the template, CuNPs can also be controlled preparation. Moreover, the size and fluorescence property of CuNPs is closely related to the length of poly(thymine). Thereafter, super-long poly(thymine) sequences have been reported to improve the fluorescence of CuNPs [27]. Through employing 2′-deoxythymidine 5′-triphosphate (dTTP) as the substrate, terminal deoxynucleotidyl transferase (TdT) has the ability to generate super-long poly(thymine) in a primer solution [27–31]. Several important targets including polynucleotide kinase [27], DNase I [28], Exo III, EcoR V [29], streptavidin [30], and DNA [31] have been detected by this amplified strategy to improve the sensitivity. Taking the advantages of convenient operation and high sensitivity, TdT-mediated super-long poly(thymine)-templated CuNP strategy expects to have good performance for facilitating fluorescent method in the field of OTA analysis.

Herein, by employing TdT generated poly(thymine) as template for fluorescent CuNP formation, we developed a fluorescence enhancement method for determining OTA concentration. As shown in Fig. 1, in the presence of OTA, OTA induced the structure of OTA aptamer switching from aptamer–cDNA duplex to G-quadruplex. Hence, cDNA released form streptavidin magnetic beads (SMB). After magnetic separation, cDNA acted as primer DNA triggering the TdT-mediated poly(thymine) formation. With poly(thymine) as templates, fluorescent CuNP can be synthesized. However, in the absence of OTA, no cDNA released, which hampered TdT polymerization. The system displayed low fluorescence. Finally, based on the fluorescence signal changes, OTA can be sensitively detected.

Experimental section

Materials and apparatus

The partly complimentary DNA strand (cDNA) of OTA aptamer with a sequence of 5’-TGTCCGATGCT-3′ and OTA aptamer with a sequence of 5′-biotin-AAAAAAAA AAAAGATCGGGTGTGGGTGGCGTAAAGGGA GCATCGGACA-3′ were purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China. https://www.sangon.com/). Ochratoxin A (OTA), zearalenone (ZEA), ochratoxin B (OTB), altenuene (ALT), aflatoxin B1 (AFB1), alternariol (AOH), tenuzonic acid (TeA), and citrinin were purchased from Pribolab Pte. Ltd. (Singapore. http://www.pribolab.com/). Streptavidin magnetic beads (SMB) (1 μm) was purchased from New England Biolabs, Inc. (England. https://www.neb.com/). Ascorbic acid was purchased from Sigma-Aldrich, Inc. (Shanghai, China. https://www.sigmaaldrich.com/china-mainland.html). 2′-deoxythymidine 5′-triphosphate (dTTP) and terminal deoxynucleotidyl transferase (TdT) were purchased from Shanghai Sangon

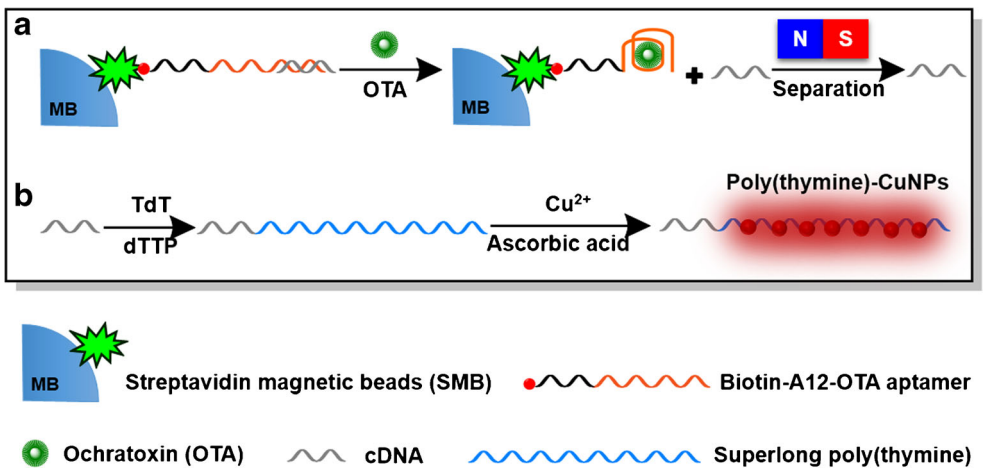

Fig. 1 Schematic illustration of the fluorescence enhancement method for OTA determination based on structure-switching of OTA aptamer and TdT-mediated super-long poly(thymine) for CuNP formation

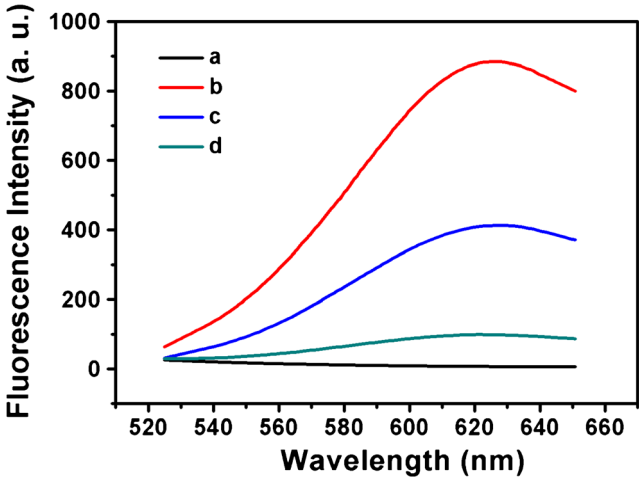

Fig. 2 Fluorescence spectra of CuNPs in the presence of different components: (**a**) cDNA + AA + Cu^{2+}; (**b**) cDNA + dTTP + TdT + AA + Cu^{2+}; (**c**) supernatant of (SMB–aptamer–cDNA + OTA) + dTTP + TdT + AA + Cu^{2+}; (**d**) supernatant of (SMB–aptamer–cDNA) + dTTP + TdT + AA + Cu^{2+}

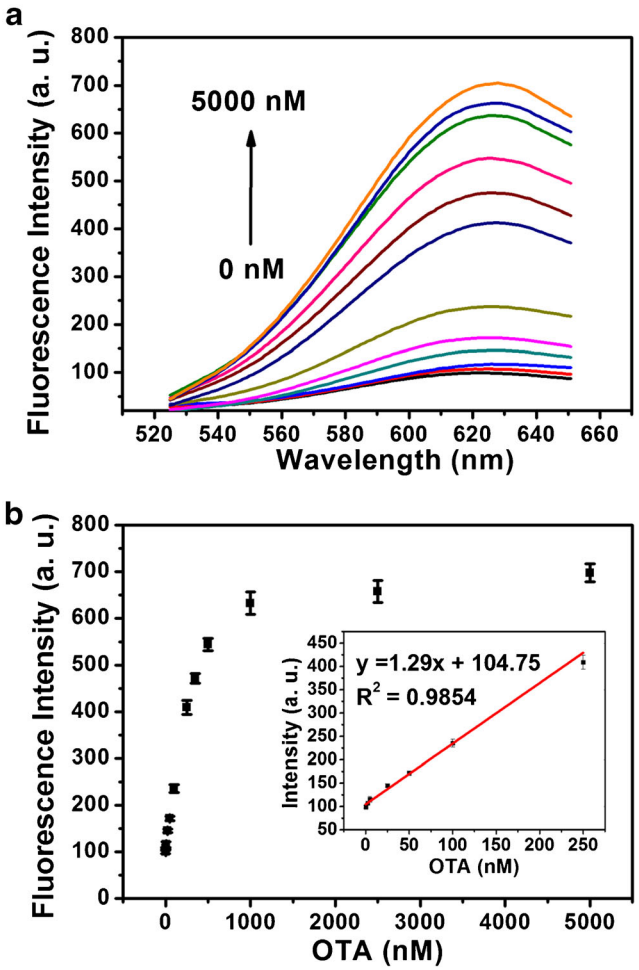

Fig. 3 **a** Fluorescence emission spectra of CuNPs under the condition of different concentrations of OTA. **b** Relationship between the fluorescence intensity of CuNPs and the concentration of OTA. The inset shows a linear relationship between the fluorescence intensity of CuNPs and the concentration of OTA from 0 nM to 250 nM ($R^2 = 0.9854$). The fluorescence intensity was monitored by measuring the emission at 625 nm. Error bars were calculated from three replicate measurements

Biotechnology Co., Ltd. (Shanghai, China. https://www.sangon.com/). Buffers used in this work: Buffer A: 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$; TdT reaction buffer: 20 mM Tris–HAc (pH 7.9), 10 mM $MgAc_2$, 0.25 mM $CoCl_2$, 50 mM KAc. Ultrapure water (18.2 MΩ·cm) was used to prepare all the solutions.

Varian cary eclipse fluorescence spectrophotometer, Varian Medical Systems, Inc. (Palo Alto, USA) was used to perform all the fluorescence measurements. The excitation wavelength was set as 340 nm. Fluorescence emission spectra were recorded in the range of 525–650 nm. The fluorescence intensity was monitored by measuring the emission at 625 nm. Software origin 8.0 was taken for the fitting of the experimental data.

Preparation of DNA-immobilized magnetic beads

Firstly, SMB were washed by buffer A. Particularly, 100 μL 4 $mg{\cdot}mL^{-1}$ solution of SMB was placed close to a magnetic scaffold for 10s. The suspension was removed and resuspended in 100 μL of buffer A. Three times buffer exchange procedure were taken place. Then, 4 μL of 100 μM biotin-aptamer in Millipore water was added to the SMB solution and well mixed for 30 min at room temperature. After that, the SMB were washed three times using buffer A to remove excess biotin-aptamer. Later, 4 μL of 100 μM cDNA in ultrapure water was added to the SMB solution and incubated for 30 min at room temperature. After five times washing by TdT buffer, excess cDNA was removed. After that, the DNA-immoblized SMB was prepared.

OTA-mediated structure-switching of aptamer

For quantitative measurement of OTA, various concentrations of OTA were mixed with 30 μL of the DNA-immoblized SMB. The mixture was incubated for 30 min at room temperature. After magnetic separation, 20 μL of the supernatant was taken for the subsequent steps of TdT-mediated polymerization and CuNP preparation. All the experiments were repeated for three times.

For OTA assay in red wine, we first diluted the red wine for 50 times by TdT buffer. After that, spiked samples were prepared by the addition of different amount of OTA to the diluted red wine. All the experiments were repeated for three times.

Table 1 Comparison reported methods for OTA determination

Method	Material	Linear range	LOD	Ref.
Electrochemical	Mercapto-β-cyclodextrin	0.01–10.0 ng·mL^{-1}	3 pg·mL^{-1}	[8]
Colorimetry	HPR	0.05–0.5 μM	10.8 nM	[9]
	Au NPs	32–1024 ng·mL^{-1}	20 ng·mL^{-1}	[10]
	DNAzyme	NR[a]	1 nM	[11]
	Phenolphthalein	10–250 ng·mL^{-1}	NR[a]	[12]
SERS	Gold film	pM–μM	NR[a]	[13]
LFA	Cy5	1–1000 ng·mL^{-1}	0.40 ng·mL^{-1}	[14]
Fluorometry	QDs	1–1000 ng·mL^{-1}	1.0 ng·mL^{-1}	[15]
	Single-walled carbon nanohorn	10–200 nM	9.8 nM	[16]
	SYBR Gold	20–500 nM	16.5 nM	[17]
	MoS_2 and QDs	1.0–1000 ng·mL^{-1}	1.0 ng·mL^{-1}	[15]
	CuNPs	0–100 ng·mL^{-1}	5 ng·mL^{-1}	[18]
	CuNPs	2.5–250 nM	2.0 nM (0.81 ng·mL^{-1})	TW [b]

[a] *NR* stands for "Not reported", [b] *TW* stands for "this work"

Terminal deoxynucleotidyl transferase (TdT) mediated polymerization

TdT polymerization was taken place by the following steps: 20 μL of the supernatant was taken and added to a solution containing dTTP and TdT. The mixture was incubated for 2.5 h at 37 °C. This reaction was terminated by heating 5 min at 85 °C.

Preparation of copper nanoparticles (CuNPs)

For CuNP preparation, ascorbic acid (4 mM) and $CuSO_4$ (200 μM) were subsequent added to above solution. After 5 min incubation at room temperature, the fluorescence spectra were recorded.

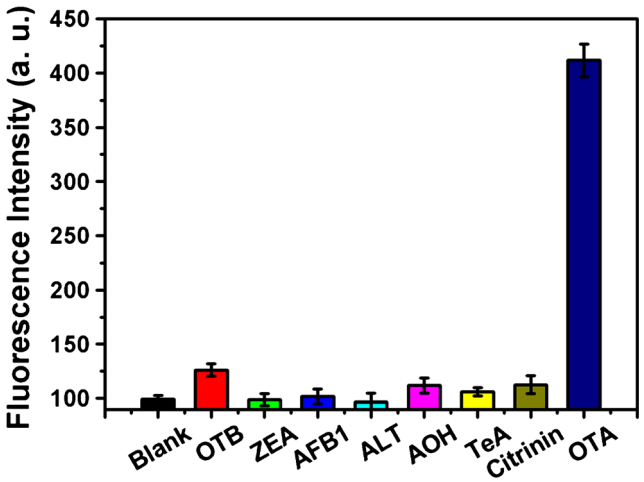

Fig. 4 Fluorescence intensity of CuNPs of the proposed method with different mycotoxins: blank control, OTB, ZEA, AFB1, ALT, AOH, TeA, citrinin and OTA. The fluorescence intensity was monitored by measuring the emission at 625 nm. Error bars were calculated from three replicate measurements

Results and discussion

Preparation of DNA-immobilized magnetic beads

As shown in Fig. 1, the biotin–A12–aptamer is immobilized onto streptavidin magnetic beads (SMB) by means of streptavidin–biotin binding. To obtain the binding amount of the biotin–A12–aptamer on SMB, OliGreen (OG) was introduced. OG is a commercially available asymmetrical cyanine dye. OG exhibits very week fluorescence. Upon binding with ssDNA, the fluorescence increased 100-fold. The maximum excitation and emission wavelength are 480 nm and 520 nm, respectively. As shown in Fig. S1, when OG is added into the biotin–A12–aptamer solution, OG–biotin–A12–aptamer complex formed immediately, resulting in a significate fluorescence enhancement. A linear relationship between the fluorescent signal and the concentration of biotin–A12–aptamer is observed. To calculate the coupling amount of biotin–A12–aptamer on MBs, the supernatant was taken to measure the fluorescence intensity in OG solution. Then the fluorescence was compared with above linear equation for quantification. As a result, the coupling amount was estimated to be 440 pmol per mg of SMB. This was further divided by the SMB number (1.35×10^9 per mg) to obtain the absolute number of biotin–A12–aptamer coupling on each SMB. As a result, it was calculated that the number was about 1.96×10^5. Thereafter, we use the same method to calculate the binding amount of the partly complimentary DNA strand (cDNA). The result was found to be 400 pmol per mg of SMB and 1.78×10^5 of cDNA on each SMB.

Feasibility validation of this strategy

The target specific structure switching of the aptamer in the presence of OTA can cause the cDNA to release and obtain

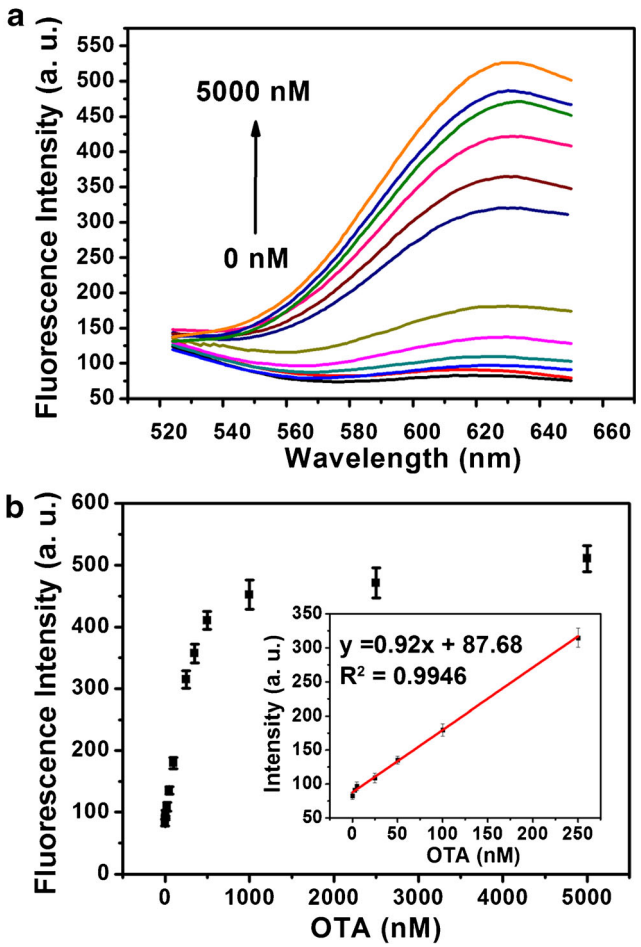

Fig. 5 **a** Fluorescence emission spectra of CuNPs under the condition of different concentrations of OTA from 0 nM to 5000 nM in 2% red wine solution. **b** Relationship between the fluorescence intensity of CuNPs and the concentration of OTA. The inset shows a linear relationship between the fluorescence intensity of CuNPs and the concentration of OTA from 0 nM to 250 nM ($R^2 = 0.9946$) in 2% red wine solution. The fluorescence intensity was monitored by measuring the emission at 625 nm. Error bars were calculated from three replicate measurements

fluorescence signal by the terminal deoxynucleotidyl transferase (TdT) generated poly(thymine) for copper nanoparticles (CuNPs) formation. To demonstrate the feasibility of this method, the fluorescence emission spectra of CuNPs which produced by cDNA and TdT-mediated polymerization products were measured, respectively. As shown in Fig. 2, curve a, neglectable fluorescence signal can be detected when using cDNA as a template. This is attributed to lack of thymine base of cDNA. However, the fluorescence intensity of CuNPs increased significantly (curve b) after introducing both TdT and dTTP into cDNA solution. This implies that TdT can mediate generation of poly(thymine) templates when using cDNA as a primer. For OTA detection, we immobilized OTA aptamer and cDNA on SMB to form SMB–aptamer–cDNA complex. In the presence of OTA, OTA induced the structure of OTA aptamer switching from aptamer–cDNA duplex to G-quadruplex. The released cDNA was employed as primer to initiate the TdT-assisted polymerization, yielding poly(thymine). A strong fluorescence was observed after the addition of Cu^{2+} and ascorbic acid because of the valid templates of poly(thymine) for CuNPs formation (curve c). Without OTA, week fluorescence was observed (curve d). Therefore, the fluorescence signal of CuNPs can indicate the concentration of OTA.

Remarkably, Song et al. [18] reported a sensitive fluorescent OTA method based on CuNP by coupling RecJf exonuclease-catalyzed target recycling amplification. Different from this method, our work is designed to improve the sensitivity from the perspective of TdT-mediated polymerization.

Optimization of method

The following parameters were optimized: (a) concentration of TdT; (b) amount of dTTP; (c) concentration of Cu^{2+}; (d) concentration of ascorbic acid; and (e) reduction time. Respective data and Figures are given in the Electronic Supporting Material. The following experimental conditions were found to give best results: (a) Optimal concentration of TdT 0.2 $U \cdot \mu L^{-1}$; (b) optimal concentration of dTTP 3 mM; (c) optimal concentration of Cu^{2+} 200 μM; (d) optimal concentration of ascorbic acid 4 mM; and (e) optimum reduction time of 5 min.

Determination of OTA

The dependence of CuNPs emission intensity on different concentrations of OTA was further investigated. With the increasing concentration of OTA, more cDNA was released. The released cDNA was employed as primer to initiate the TdT mediated polymerization. Thus, the fluorescence signals of CuNPs increased gradually with increasing OTA concentration. From Fig. 3, there is a good linear relationship between the fluorescent signal and the concentration of OTA from 2.5 nM to 250 nM. The linear equation is $y = 1.29x + 104.75$, where y indicates the fluorescence intensity at 625 nm and x denotes the concentration of OTA. The limit of detection (LOD) was 2.0 nM by the definition of the $3S_0/S$ (S is the slope of the linear equation, and S_0 is the standard deviation for the blank solution, $n = 11$). In the European Union, the maximum threshold of OTA allowed in wine is 2 $\mu g \cdot kg^{-1}$ [32], which is higher than the detection limit of our method. Besides, the analytical performance of our method is comparable with most of the previous reported methods (Table 1).

Selectivity study

To demonstrate the specificity of this method, some other mycotoxins such as ochratoxin B (OTB), zearalenone (ZEA), aflatoxin B1 (AFB1), altenuene (ALT), alternariol (AOH), tenuzonic acid (TeA), and citrinin were tested

respectively. As shown in Fig. 4, OTB, ZEA, AFB1, ALT, AOH, TeA and citrinin induce negligible fluorescence changes. However, OTA exhibits a stronger fluorescence response, prominently. Therefore, this method shows outstanding specificity towards OTA assay.

Performance in 2% red wine

We further challenged the practicability of this method by performing the detection of OTA spiked in red wine samples. 50-fold-diluted red wine was taken as the complicated sample matrix. When OTA was spiked in the diluted red wine, this method still shows sensitive response to OTA. As we can see in Fig. 5, with the increasing concentration of OTA, the fluorescence signals of CuNPs still increased in the complex environment. Besides, a similar linear range was exhibited at the concentration from 2.5 nM to 250 nM with $R^2 = 0.9946$. The results suggested that this method might be feasible for OTA assay in real complex samples. It is worth noting that comparing this method in the clean buffer for OTA assay with that in the diluted red wine, the fluorescence intensity is smaller. This phenomenon may result from the interference of the complex molecules in red wine for CuNP formation (Fig. S7). Since the measurements were repeated for three times independently for each concentration of OTA, the average relative standard deviation is calculated to be 5.02%, which is acceptable. In addition, to investigate the accuracy of the proposed method, four 50-fold-diluted red wine samples spiked with different concentrations of OTA were measured by high performance liquid chromatography (HPLC) and the proposed method. As shown in Fig. S8, there is a linear relationship between the results from these two methods. Therefore, the accuracy of the proposed method is acceptable.

Conclusions

In summary, on the basis of terminal deoxynucleotidyl transferase generating polythymine for CuNP synthesis, a novel ochratoxin A (OTA) fluorescent aptasensor has been developed. Since poly(thymine)-scaffolded CuNPs possess easy preparation procedure, and TdT-mediated polymerization can directly amplify the signal, the proposed method is convenient and sensitive. Besides, this method extends the application of TdT generated poly(thymine)-CuNP-based strategy for small molecules determination. Moreover, this method was designed in terms of structure switching of OTA's aptamer. Therefore, it has the potential for other target detection through replacing a corresponding aptamer.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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