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ORIGINAL PAPER

In-situ amplified voltammetric immunoassay for ochratoxin A by coupling a platinum nanocatalyst based enhancement to a redox cycling process promoted by an enzyme mimic

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Abstract A new signal amplified protocol for sensitive monitor of ochratoxin A was developed by coupling platinum enhancement technique to a redox cycling amplification strategy. Initially, platinum-enclosed gold cores (AuPtNP) were functionalized with monoconal antibody against ochratoxin A (OTA) to act as signal tags. Upon addition of analyte (OTA), competitive immunobinding occurs between OTA and an OTA-BSA conjugate immobilized on a ferrocene modified electrode for the anti-OTA on the signal tags. Next, the AuPtNPs on the immunosensor are incubated with a platinum enhancing solution to initiate the growth of additional catalysts in order to further promote the catalytic cycling between p-aminophenol and p-quinoneimine with the aid of the reductant $NaBH_4$ and ferrocene. As a result, the analytical signal is strongly enhanced and can be measured by differential pulse voltammetry in the range from −300 mV to 600 mV (vs. SCE) at 50 mV s^{-1}. Under optimized conditions, the immunosensor displays a dynamic working range that extends from 0.2 pg·mL^{-1} to 5 ng·mL^{-1} of OTA, with a lower detection limit of 75 fg·mL^{-1}. The method is highly selective and was applied to the determination of OTA in (spiked) red wine samples.

Keywords Ochratoxin A · Competitive immunosensor · Ferrocene · Enzyme mimic · Cyclic voltammetry · Food safety · AuPt nanoparticles · Platinum enhanced process · Redox cycling · Catalytic strategy

Introduction

Ochratoxin A (OTA), produced mainly by *Aspergillus Ochraceous*, *A. carbonarius* and *Penicillium verrucosum* is a carcinogenic in human (group 2B) [1], is nephrotoxic, carcinogenic and teratogenic, and regarded as an immunosuppressive agent [2]. It can be found in various agricultural commodities during storage, such as grape juice, baby food, beans, soluble coffee and wine [3, 4]. Thus, numerous studies have been reported for reliable and sensitive analysis of OTA in food samples. Methods including liquid chromatography-tandem mass spectrometry, high-performance liquid chromatography, gas chromatography and thin-layer chromatography have been adopted for sensitive detection of OTA [5–7]. However, more convenient, less expensive and faster detection methods are required in daily life. Multiple immune-biosensors have emerged for OTA detection including photoelectrochemical, electrochemical, electrochemilu minescent, and fluorescent immunosensors [8–11]. Among these methods, electrochemical approaches have drawn significant attentions and been considered as effective techniques for OTA detection.

Detectable signal amplification is especially important in increasing the approach sensitivity. Enzymatic and nano amplification are most frequently used signal amplification approaches [12–14]. Many nanocatalysts especially noble

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metals have been widely used to improve the detection sensitivity in immunosensor due to its intrinsic advantages including eco-friendly, highly active and low cost [15–18]. Platinum-based catalysts have been extensively developed as efficient catalysts towards many reactions, e.g. oxygen reduction reaction, hydrogen evolution, and methanol oxidation reaction [19–21]. Multiple immunosensors have been developed based on platinum nanocatalyst promoted signal amplified strategy, e.g. catalytically oxidize 1-NP, catalyzed the reduction of hydrogen, electro-oxidation of hydrazine, and platinum-catalyzed hydrogen evolution reaction [22–24].

Redox cycling signal amplified protocol, a process that can repetitively generate or consume signaling species, has attracted vast attentions in the last few years. Generally, enzymatic and non-enzymatic catalysis are usually combined with redox cycling signal amplified process for enhancing the detection signal in electrochemical immunosensor [25, 26]. Especially, platinum nanoparticles as an efficient catalyst are regarded as a proper candidate for promoted redox cycling signal amplified strategy. The author has devised an immunosensor by combining the platinum nanoparticles based redox cycling with *in situ* signal amplification strategy [27]. In the amplified assay, the self-produced reactant (e.g. *p*-aminophenol) can be catalytically recycled by platinum nanoparticles, thus resulted in the signal amplification. However, we found that the catalytic activity of the nanocatalysts was greatly weakened when they labeled with biomolecules. The reason might be speculated that some of the active sits of the platinum nanoparticles may be covered after labeling with biomolecules. Moreover, the biomolecules is weak conductive, which may influence the electron transfer from the detection solution to the working electrode.

The platinum enhancement technique can be considered as ideal alternative strategy to increase the signal. The enhancement step is required to allow for seed-mediated Pt deposition on the biomolecules surface in order to increasing the catalytic active sites of the nano-labels [28]. Thus, in the assay, a new and sensitive electrochemical immunosensor was developed for monitoring of small molecules (ochratoxin A, OTA, as a model) based on Pt nanocatalyst promoted redox cycling signal amplification strategy coupling with Pt enhancement step. Platinum-enclosed gold cores nanocomposites (AuPtNP) were synthesized and used as signal tags. After competitive immune-reaction, the signal tags were immobilized on the ferrocene-modified glassy carbon electrode. Then, multiple Pt catalyst was grown on the surface of the signal tags after performing Pt enhancement step, which can significantly catalyze the reduction of *p*-nitrophenol to *p*-aminophenol in the presence of $NaBH_4$. Moreover, with the aid of Fc and Pt nanocatalyst, the PAP can be generated recycled, thus resulted in the signal amplification.

Experimental

Reagents

Ochratoxin A (OTA), OTA-BSA conjugated were purchased from Sigma-Aldrich (Shanghai, China www.sigmaaldich.com/china). Monoclonal antibody anti-OTA was supplied by Immunechem (Burnaby, Canada www.immunechem.com). $NaBH_4$, *p*-nitrophenol (PNP), *p*-aminophenol (PAP) were obtained from Sinopharm Chem. Re. Co. Ltd. (Shanghai, China www.sinoreagent.com). Chitosan (deacetylating grade: 80–95%), glutaraldehyde, formic acid, ferrocenecarboxylic acid and bovine serum albumin (BSA, 96–99 wt%) were purchased from Sigma-Aldrich (Shanghai, China www.sigmaaldich.com/china). All other reagents were of analytical grade and were used without further purification. Distilled water was used throughout the study and phosphate-buffered saline (PBS, 0.1 M) of various pHs were prepared by mixing of the stock solutions of Na_2HPO_4 and NaH_2PO_4.

Preparation and biolabeling of platinum enclosed gold nanoparticles (AuPtNPs)

Initially, gold nanoparticles were synthesized according to the literature with a little modification [29]. Briefly, the pre-cooled $HAuCl_4$ (0.4 mM) and ascorbic acid (0.64 mM) with the same volume were mixed rapidly into a flask. Then keep it in an ice bath until the color turned to vivid red. The gold colloids were stored at room temperature for further use.

Then, 2 mL of hexachloroplatinic acid (2.5 mM) and ascorbic acid (38 mM) were added in the above gold colloids, respectively. The resulting mixtures were reacted for 4 h at 70 °C. Subsequently, 4 mL of ascorbic acid was added and temperature was raised to 80 °C. Two hours later, the mixture was centrifuged and the AuPtNPs was redispersed in deionized water for further use. Subsequently, the AuPtNPs solution was used for the conjugation of *anti*-OTA antibody as follows: 400 μL of *anti*-OTA (1.0 mg mL^{-1}) was initially added into 1.0 mL of AuPtNPs colloids, and then the mixture was incubated for 12 h at 4 °C with gentle stirring. Finally, anti-OTA functionalized AuPtNPs were obtained by centrifugation, and redispersed.

Fabrication of OTA immunosensor

Firstly, chitosan solution was prepared by adding 1.0 mg of chitosan flakes into 1.0 mL of 1.0 wt% acetic acid solution, and then the mixture was kept stirring until the flake was completely dissolved. Then, 1.0 mg of ferrocenecarboxylic acid was added into the chitosan solution with adequately stirring until a homogeneous mixture was obtained

(designated as Chi-Fc). Following that, 10 μL of the Chi-Fc solution was dropped on the surface of the pre-treated GCE. After dry, it was rinsed in pH 7.4 PBS. Afterwards, 10 μL drop of glutaraldehyde (0.020%, *w*/*v*) was applied to the GCE surface and incubated for 1 h. Excess glutaraldehyde was removed after rinsing in pH 7.4 PBS. Subsequently, the modified electrode was incubated with the OTA-BSA solution (5 μg mL^{-1}) or 12 h at 4 °C. Then the electrode was immersed in the blocking solution (2.5 wt% BSA) for 1 h at RT to block the possible active sites on the surface of the electrode. The electrode was stored at 4 °C when not in use.

Electrochemical measurement

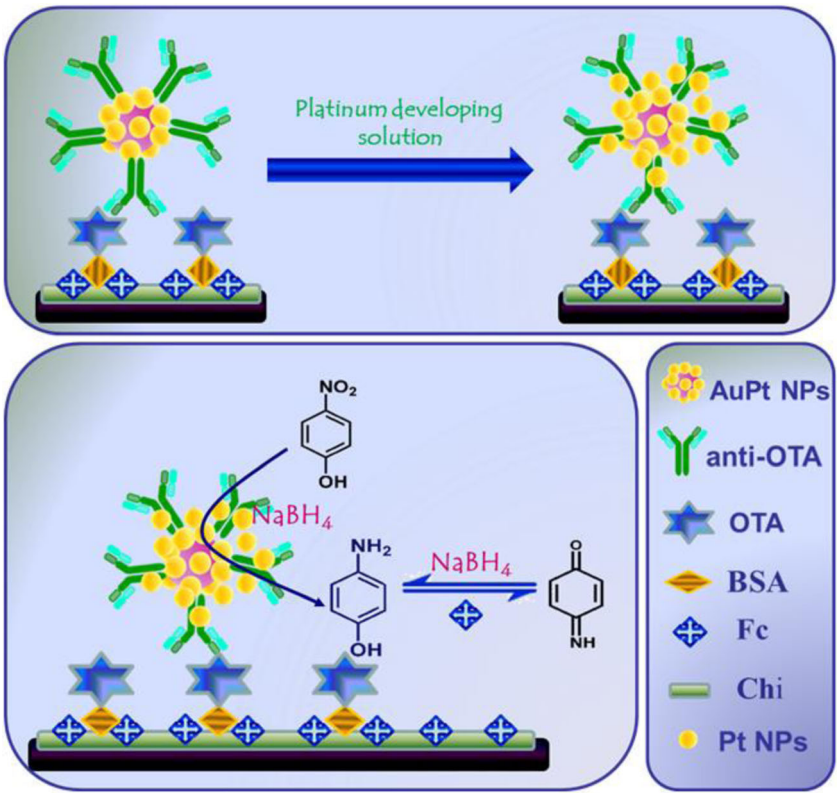
To carry out the competitive immunoreaction and electrochemical determination, firstly, 5-μL of OTA standard solutions or wine samples with various concentrations and 5-μL of *anti*-OTA-AuPtNPs (~0.1 mg mL^{-1}) were dropped onto the modified electrode and incubated for 23 min at RT. After washed with the washing buffer, the resulting immunosensor was reacted with the platinum enhance solution containing 2.0 mM $PtCl_4^{2-}$, 0.5% of Tween 80 and 0.1 M formate for 40 min. After that, the immunosensor was monitored in pH 7.4 PBS solution containing 1.0 mM PNP and 1.0 mM $NaBH_4$. Analyses are always made in triplicate (Scheme 1).

Results and discussion

Characterization of signal amplified mechanism of the assay

In this work, platinum-enclosed gold cores nanocomposites, synthesized and used as signal tags, can trigger the Pt enhancement step. Thus, multiple Pt nanoparticles were in situ generated on the signal tags, which can induce the *p*-aminophenol redox cycling and greatly enhance the electrochemical signal. As mentioned above, one important concern for the OTA-sensing strategy was whether AuPtNPs-based labels can trigger the Pt enhancement process. To verify this point, transmission electron microscope (TEM) was employed. From TEM images of AuPtNPs (Fig.1a), a uniform and flower-like shape was observed with an average size of 40 nm. In addition, it is obviously that the thickness and size of nanoparticles was increased and more small particles was grown on the edge of the signal labels after performing with platinum enhancing solution (Fig.1b). Thus, we can preliminary observed that the Pt enhancement step was successfully performed.

Another important concern was whether the nanocomposite can catalyze the *p*-nitrophenol reduction with the aid of $NaBH_4$. The reduction process can be monitored by UV-vis absorption spectra. To realize our design, $NaBH_4$ was added with an excess amount. As indicated from the Fig.1c, with the reaction proceeds, the absorbance at 402 nm (the peak of *p*-nitrophenolate



Scheme 1 Schematic illustration of sensitive OTA electrochemical immunosensor based on platinum enhancing process and nano-zyme promoted redox cycling amplified strategy

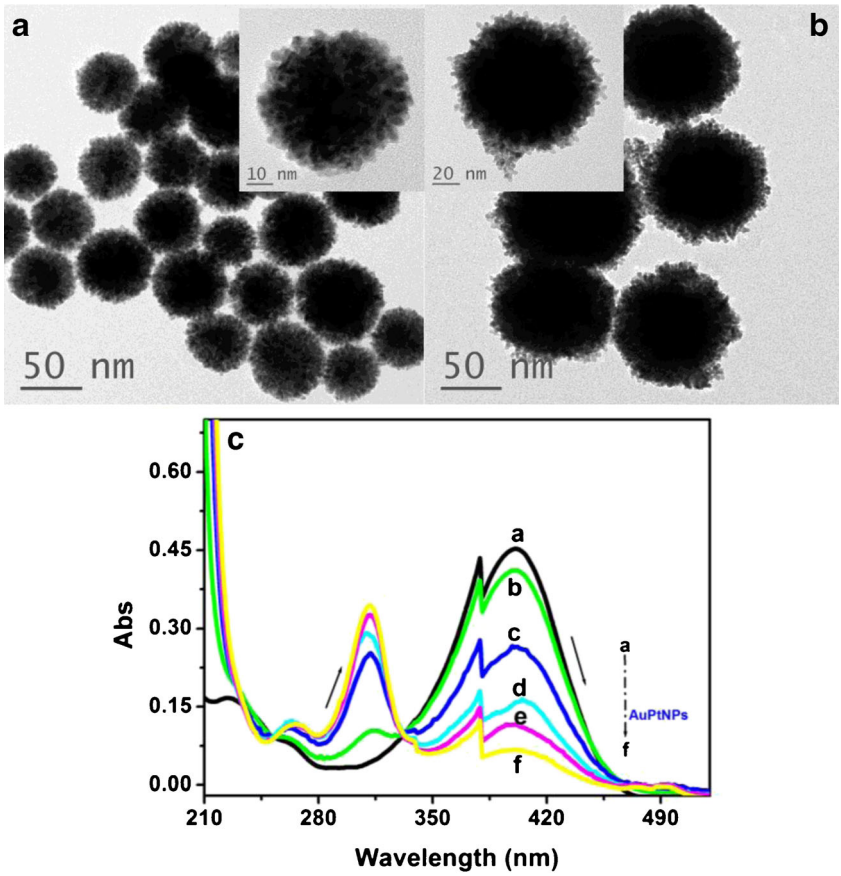


Fig. 1 TEM image of **a** AuPtNPs-*anti*-OTA, **b** substrate ′A′ after reaction with platinum enhance solution. **c** UV-vis absorption spectra of (*a*) the *p*-nitrophenol solution in the presence of $NaBH_4$, and (*b-f*) the mixture 'a' after addition of various concentrations of AuPtNPs nanocatalysts

ions) was gradually decreased with the increment of nanocomposite, while that peak at 290 nm (the peak of *p*-aminophenol) was increased. Thus, the results indicated that the AuPtNPs showed excellent catalytic activity towards the reduction of PNP.

Electrochemical characteristic the recycling of self-product and the Pt enhancement process

Logically, one puzzling question arises as to whether the Fc-modified electrode can promote the redox cycling of PAP to PQI. To clarify this issue, the sensor was initially monitored in a pH 8.0 phosphate buffer solution. As indicated in Fig. 2a, a couple of redox peaks was observed. After addition of 6 mM PAP, the peak current of electrode was obviously increased. The reason might be attributed to the fact that the PAP can be electrochemical oxidized via an electron mediation of the electron mediator (ferrocene) on the electrode. Moreover, the signal was greatly increased after the addition of $NaBH_4$ to the PBS. Thus, it is observed that the PAP can be recycled with the aid of the electron mediator and $NaBH_4$.

Following that, the OTA-BSA modified electrode was used for the reaction with OTA (0.001 ng mL^{-1}) and AuPtNPs-*anti*-OTA. Electrochemical behaviors of the formed immunosensor were monitored in the 8.0 PBS with and without the PNP and $NaBH_4$. As indicated from the Fig. 2b, when PNP and $NaBH_4$ was added in the detecting solution, the peak current was greatly increased, which cause a 561% signal increase in the anodic current relative to the background current. The reason might be the fact that the AuPtNPs can reduce the PNP with the aid of $NaBH_4$, and the self-product PAP can promote the redox cycling of PAP to PQI. Interestingly, the peak current was further highly increased after the immunosensor incubation with the platinum enhancing solution. The reason might be speculated that (i) more platinum nanoparticles can be generated *in situ* on the surface of the immunosensor, (ii) the catalytic active sites on the labels was increased after performing platinum enhancing process, and (iii) the amplified strategy can be applied for sensitive detection of target.

Conditional optimization

To achieve the best performance of the AuPtNPs based electrochemical immunosensor, several experimental conditions including incubation time, the reaction time for platinum enhancing, the concentration of ferrocene, the concentration of $PtCl_4^{2-}$ in platinum enhancing solution and the concentration of $NaBH_4$ were investigated. Considering the possible

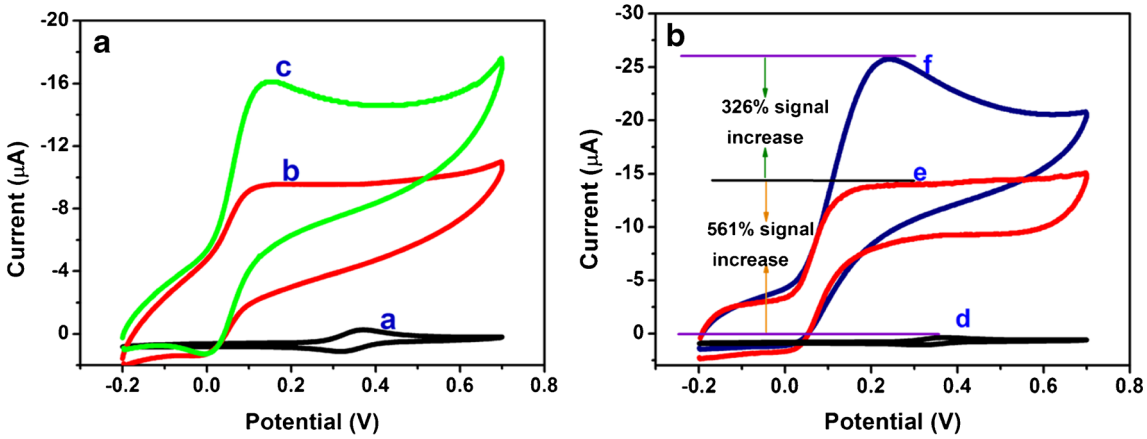


Fig. 2 **a** Cyclic voltammograms of CS-Fc-modified GCE in (*a*) pH 8.0 PBS, (*b*) pH 8.0 PBS + 6.0 mM PAP, and (*d*) pH 8.0 PBS + 6.0 mM PAP + $NaBH_4$ at 50 mV/s. **b** Cyclic voltammograms of the AuPtNPs-*anti*-OTA/OTA-BSA-Cs-Fc-GCE in (*a*) pH 8.0 PBS, pH 8.0 PBS + $NaBH_4$ + 6.0 mM PNP, and (*c*) the immunosensor after reaction with platinum enhance solution in pH 8.0 PBS+ $NaBH_4$ + 6.0 mM PNP at 50 mV/s

application of the electrochemical immunosensor in the future, room temperature (25 °C) was selected for immunoreaction throughout the experiment. At the condition, the effect of the incubation time for antigen-antibody interaction was investigated. As depicted in Fig.3a, the peak current decreased with the increasing of the reaction time from 5 min to 23 min, and tended to level off after 23 min. Thus, 23 min was selected for sensitive determination of OTA at acceptable throughout.

As described above, the amount of the enhanced platinum nanoparticles on the immunosensor was one of important factors to determine the signal enhancement. Thus, the reaction time of platinum enhancing process was also investigated. Initially, the immunosensor was incubation with 0.01 ng mL^{-1} OTA and bio-AuPtNPs mixture for 23 min at RT, and then reaction with platinum enhancing solution with different times. As indicated from Fig.2b, with the increasing reaction

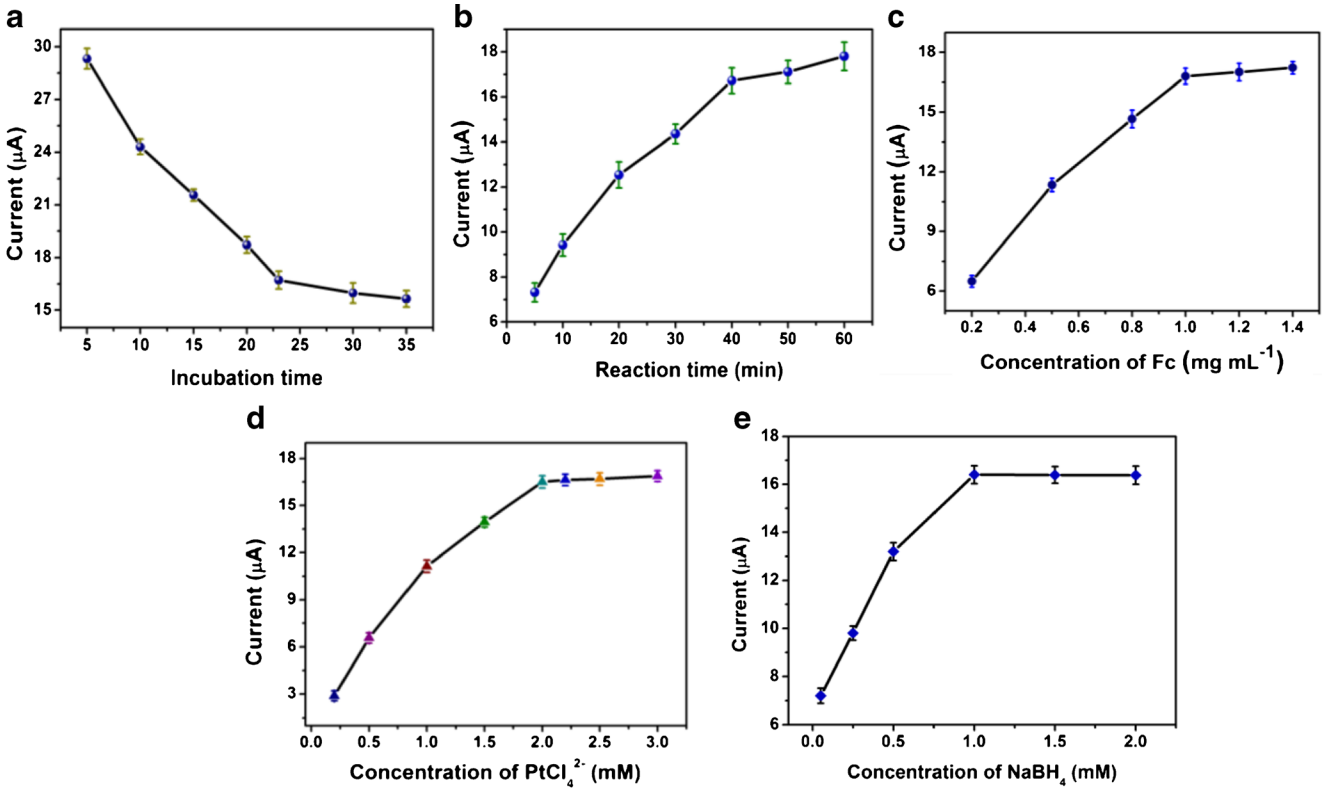


Fig. 3 Effects of (**a**) incubation time, (**b**) formation time of Pt catalysts on the response of the impedimetric immunosensor, and the concentration of Fc (**c**), $PtCl_4^{2-}$ (**d**), $NaBH_4$ (**e**)

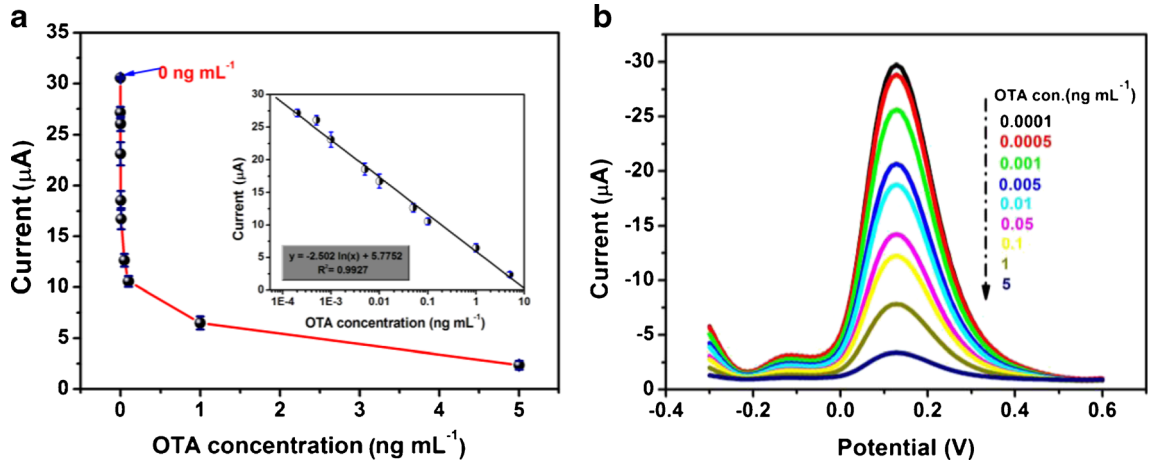


Fig. 4 **a** Calibration plots of the electrochemical immunosensor toward different concentrations OTA standards in pH 8.0 PBS + $NaBH_4$ + 6.0 mM PNP (peak currents at 128 mV were used for quantity); **b** the corresponding DPV curves

time, the currents were increased. It was tended to a plateau at 40 min. Therefore, 40 min was selected for platinum development. Similarly, we observed that the currents increased with the increase of ferrocene concentrations and a highest signal can be achieved when 1.0 mg mL^{-1} of ferrocene was used (Fig. 2c). Moreover, as indicated from Fig. 2d and e, 2.0 mM of $PtCl_4^{2-}$ and 1.0 mM $NaBH_4$ were chosen as the optimal condition for platinum enhancing and PNP reduction.

Analytical performance of the OTA-based electrochemical sensor

By virtue of the optimal conditions, the analytical properties of the competitive-type electrochemical immunoassay were evaluated toward OTA standards. The incubation solution contained an OTA standard at a known concentration and AuPtNPs-*anti*-OTA substrate at a certain concentration. The Pt nanocatalyst can generated on the Fc-OTA-BSA-modified electrode, which can accelerate the catalyze reduction of *p*-nitrophenol and thus inducing the redox cycling of PAP. As seen from the figure, the peak currents decreased with the increase of OTA concentration in the sample, and exhibited a good linear relationship between the peak currents and the logarithm of OTA concentrations in the dynamic range of 0.2 pg mL^{-1}- 5 ng/mL (Fig. 4a). The linear regression equation was $y = -2.502\ln(x) + 5.7752$ ($R^2 = 0.9927$). The limit of detection was 0.075 pg mL^{-1}.

Moreover, the analytical properties of the AuPtNPs based redox cycling sensor was compared with other OTA sensors [3, 18, 30–35]. As indicated from Table 1, the analytical performance of the sensor was acceptable (Table 2).

Precision, stability and selectivity of the OTA based electrochemical sensor

In order to evaluate the specificity of the redox-cycling based electrochemical immunosensor toward target OTA, the system was challenged with other mycotoxins,

Table 1 Comparison of analytical properties of the AuPtNPs based OTA assay with other methods

Assay method [a]	Materials used [a]	Linear range	LOD	Ref.
DPV assay	None	0.005–100 ng mL^{-1}	2 pg mL^{-1}	[30]
FRET-based competitive biosensor	CdTe QDs; Magnetic silica NPs	8.0–48 pg mL^{-1}	0.8 pg mL^{-1}	[3]
Amperometric aptasensor	SWCNTs	0.18–30 nM	52 pM	[31]
LRET based aptasensor	$NaYF_4$:Yb, Er NPs; AuNRs	0.05–100 ng mL^{-1}	27 pg mL^{-1}	[18]
Fluorescence immunoassay	CdTe QDs	0.05–10 pg mL^{-1}	0.05 pg mL^{-1}	[32]
Colorimetric aptasensor	Au@Fe_3O_4 NPs	0.5–100 ng mL^{-1}	30 pg mL^{-1}	[33]
Electrochemical aptasensor	$mSiO_2$@Au; GAu/CdTe	0.0002–4 ng mL^{-1}	0.07 pg mL^{-1}	[34]
Impedimetric aptasensor	modified PPy-dendrimers	-	2 ng L^{-1}	[35]
Electrochemical sensor	AuPtNPs	0.0002–5 ng mL^{-1}	0.075 pg mL^{-1}	This work

[a] *DPV* Differential pulse voltammetric, *QDs* quantum dots, *NPs* nanoparticles, *SWCNTs* single-walled carbon nanotubes, *LRET* luminescence resonance energy transfer, *AuNRs* gold nanorods, *$mSiO_2$@Au* gold nanoparticles functionalizd silica-coated iron oxide magnetic nanoparticles, *GAu/CdTe* cadmium telluride quantum dots modified graphene/Au nanocomposites, *PPy* polypyrrole

Table 2 Determination of OTA in red wine sample with the redox cycling amplified immunosensor ($n = 3$)

Sample	Added (ng mL^{-1})	Assayed (ng mL^{-1})	RSD (%)	Recovery (%)
1	0.005	0.00472	2.9	94.4
2	0.010	0.0109	4.3	109.0
3	0.100	0.095	3.3	95.0
4	0.500	0.498	2.6	99.6
5	1.000	0.976	2.8	97.6
6	2.000	2.10	3.8	105.0

including aflatoxin B_2 (AFB_2), ochratoxin B (OTB), fumonisin B_1 (FB_1), aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1). The investigation was tested by incubating the sensor in interfering agent with and without target OTA. As shown in Fig. 4b, the currents observed from AFB_2, OTB, FB_1, AFB_1 and AFG_1 alone were almost the same as the background signal. Moreover, the mixture with target OTA molecules did not induce the significant change in the resistance relative to target molecules alone. Taking into consideration, the selectivity of electrochemical immunosensor was acceptable.

The stability of the electrochemical immunosensor was investigated by storing the electrode at 4 °C for 20 days. It is found that the electrochemical signal was not obvious change but only a 6.7% decrease of its initially currents after 40 days storage. The reproducibility of the electrochemical assay was also evaluated using various batches of immunosensors. The results indicated that the coefficients of variation of the inter-assay were 8.3%, 9.4% and 10.6% ($n = 3$) towards 0.01 ng mL^{-1}, 0.5 ng mL^{-1} and 5 ng mL^{-1} OTA, respectively, whereas the CVs of the intra-assay with various batches were 8.5%, 7.8% and 9.0% toward the above-mentioned analyte (Fig. 5).

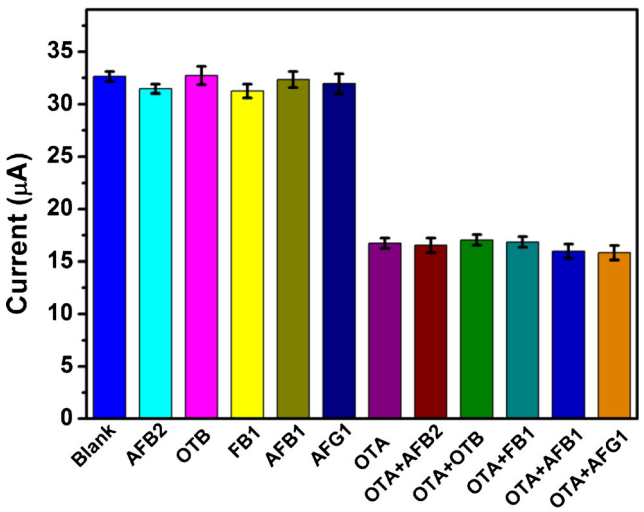


Fig. 5 The specificity of the electrochemical immunosensor towards AFB2, AFB2, AFG1, FB1 and OTB (100 ng mL^{-1})

Recovery performance

In order to evaluate the feasibility and trueness of the immunosensor for the analysis of real sample, the electrochemical immunosensor was employed for the detection of OTA in red wine samples by using recovery test (Table 1). Then, 0.005, 0.010, 0.100, 0.500, 1.000 and 2.000 ng mL^{-1} of OTA standards with various concentrations were spiked into the red wine samples and detected by the AuPtNPs-based immunosensor. As indicated from Table 1, the concentrations determined by this assay were 0.00472, 0.0109, 0.095, 0.498, 0.976 and 2.1 ng mL^{-1}. The recoveries were found to vary from 94.4 to 109.0% and the relative standard deviation (RSD) was in the range of 2.8–3.8%. The results showed that the immunosensor can be employed to monitor the target OTA in a complicated sample matrixs.

Conclusions

In summary, the work reported on a new signal-amplification electrochemical immunosensor for sensitive monitoring of OTA (a kind of mycotoxins used in the case) in complicated foodstuff, coupling platinum enhancing process with nanozyme promoted redox cycling amplified strategy. Experimental results indicated that multiple Pt nanocatalyst can generate *in situ* on the *anti*-OTA-AuPtNPs-modified electrode, which showed high catalytic properties for the catalytic reduction of PNP with the aid of $NaBH_4$. Moreover, the product PAP can be catalytic recycled with the help of the ferrocene and the reducing agent, thus leading to the highly signal amplification. With the Pt enhancing process, the signal of the redox cycling based signal amplified strategy was highly enhanced due to the fact that (i) the catalytic active sites was largely increased, (ii) more platinum nanocatalysts was grown on the electrode, which can efficient catalytic the reduction of PNP, thus induced the redox cycling amplification. Nevertheless, one disadvantage of our strategy is that the long reaction time (40 min) for the Pt enhancing process. Future work should focus on the shorting the reaction time.

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

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