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An electrochemical biosensor for the direct detection of oxytetracycline in mouse blood serum and urine

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Oxytetracycline (OTC), a broad-spectrum antibiotic, has been extensively used as a food additive for livestock. Its extensive use has greatly increased the risk of chronic drug abuse and has also increased the risk of the resulting diseases. Therefore, in light of this emerging situation, the detection of OTC in both food and livestock is very important to reduce the risks and for diagnosis purposes . In this work, we have proposed an electrochemical aptasensor to quantify OTC. The biosensor shows considerable sensitivity and selectivity, and it can be easily operated and regenerated. Furthermore, for the first time, we have shown that an electrochemical aptasensor can be directly used to detect OTC in mouse blood serum and urine. This biosensor has the potential to aid in the analysis of residual OTC levels, as well as providing more pharmacokinetic information in the future.

1 Introduction

Oxytetracycline (OTC) is a broad-spectrum antibiotic with the ability to interfere with the production pathways of essential proteins for bacteria.[1,2] In order to prevent illegal abuse of OTC, which results in drug tolerance, a temporary acceptable daily intake (ADI) and a maximum residue limit (MRL) for OTC was set in 1990 by the World Health Organisation (WHO) (ADI: 0–0.15 μg g^{-1}; MRL: 0.1 μg g^{-1} for milk, 0.2 μg g^{-1} for meat, and 0.3 μg g^{-1} for eggs).[3] Several methods including ELISA, HPLC, surface plasmon resonance, *etc.* have been developed to detect residual OTC.[4–8] However, since the discovery that OTC possesses efficacy in respiratory and gastrointestinal therapies and has an amazing positive effect on the growth of livestock,[9] this antibiotic has been widely used as a food additive for livestock in recent decades. As a result, humans have an increased chance of a long-term intake of OTC. More worryingly, recent research has shown that extensive exposure to OTC may cause gastrointestinal and photosensitive allergic reactions, especially in humans.[10,11] Therefore, the efficacy and toxicity of OTC should be re-evaluated, and thus the detection of OTC becomes much more important and urgent. However, the available traditional detection methods do not meet the new demands. The sensitivity and specificity of the traditional methods is not good enough for the detection of OTC in a real sample, and expensive instruments and tedious operations are usually required.

The recent advances in aptamer technology have provided more opportunities to propose better methods for OTC detection, and there have been developments in OTC detection using aptamer recognition.[12–15] These efforts have greatly improved the detection performance. Nevertheless, owing to the complexity of real samples, the aptamer-based detection of OTC in real samples has not been achieved so far. In view of this fact, here we propose an electrochemical aptasensor to quantify residual OTC in mouse blood serum and urine. No pretreatment of samples is required in our sensing system, and the entire detection process can be completed in a very short time, suggesting that this method has huge potential in clinical and food security applications. Furthermore, since the electrochemical apparatus is cost-effective and can be easily miniaturized, the development of high efficiency and low cost domestic medical instruments to detect OTC and some other antibiotics may be achieved in the future.

2 Experimental

2.1 Chemicals and materials

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 6-mercapto-1-hexanol (MCH), hexaammineruthenium(III) chloride ($[Ru(NH_3)_6Cl_3]$) ferrocene (Fc) and OTC were purchased from Sigma. Blood serum and urine samples were taken from Kunming (KM) female mice, which were provided by the Nanjing Lab Animal Research Center (Nanjing, China). All other chemicals were of analytical reagent grade. The oligonucleotides used in this work were synthesized from Takara Biotechnology Co., Ltd. (Dalian, China). The sequences of the synthesized oligonucleotides are:

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DNA-1: 5′-HS-$(CH_2)_6$-**GCA TGC CTT** AAG CGA TCG CCA TAT TAT **AAG GCA TGC**-Fc-3′

DNA-2 (OTC aptamer): 5′-CGT ACG GAA TTC GCT AGC GGG CGG GGG TGC TGG GGG AAT GGA GTG CTG CGT GCT GCG GGG ATC CGA GCT CCA CGT G-3′

The sequences of DNA-1 shown in bold are complementary sequences, which can self-fold with the help of Mg^{2+} to form a hairpin structure in appropriate conditions.

Double-distilled water was purified to a specific resistance of >18 MΩ cm with Millipore System and used to prepare all the solutions except OTC (diluted in ethanol). The buffer solutions employed in this work are as follows. DNA immobilization buffer: 10 mM tris–HCl, 1 mM EDTA, 10 mM TCEP, and 0.1 M NaCl (pH 7.4). Hybridization buffer: 120 mM tris–HCl, 100 mM NaCl, 2 mM $MgCl_2$ and 5 mM KCl (pH 7.4). Stabilization buffer: 0.5 M $NaClO_4$, 10 mM HEPES (pH 7.0).

2.2 The preparation of the DNA-functionalized gold electrode

The gold electrode (3 mm diameter) was firstly immersed in piranha solution (98% H_2SO_4: 30% H_2O_2 (V : V) = 3 : 1) for 10 min *(Caution: piranha solution reacts violently with organic solvents and should be handled with great care!)*. Then, it was rinsed with double-distilled water. After that, the electrode was carefully polished using P5000 silicon carbide paper and an alumina slurry (1 μm, 0.3 μm, 0.05 μm), in sequence. Then, it was ultrasonically washed in both ethanol and double-distilled water for about 5 min to remove impurities. Finally, the electrode was electrochemically cleaned with 0.5 M H_2SO_4 to remove any remaining impurities. After being dried with nitrogen, the electrode could be used for DNA-1 immobilization.

The gold electrode substrate was incubated with DNA-1 (1.0 μM in an immobilization buffer) at 4 °C for 16 h to form a self-assembled DNA monolayer on the surface of the electrode. DNA-1 had been pretreated by heating to 95 °C and cooling down rapidly in ice to unfold the DNA chains. The DNA-1 modified electrode surface was then passivated with 1 mM MCH for 0.5 h to reduce nonspecific interactions between the DNA probe and the surface as well as to stabilize the self-assembled monolayer. After that, the immobilized DNA-1 was allowed to hybridize with DNA-2 (1.0 μM in a hybridization buffer) at room temperature for 2 h to form double-stranded DNA (dsDNA), followed by a final treatment with the stabilization buffer for 10 min to unify the valence of the Fc probe, which was labeled at the 3′-end of the DNA-1. At this point, a DNA-functionalized gold electrode had been prepared.

2.3 The preparation of blood serum and urine samples

All animal procedures were performed in accordance with institutional and national guidelines and with approval from the Animal Care Ethics Committee of Nanjing University. Female KM mice with similar weights were housed in standard cages. They were starved of food and water for 12 h before oral administration of 10 μg g^{-1} OTC. The KM mice were killed 3.5 h after dosing. Blood serum was collected by centrifugation, while urine was obtained without further treatment. The samples were immediately used in the OTC assay or stored at −20 °C.

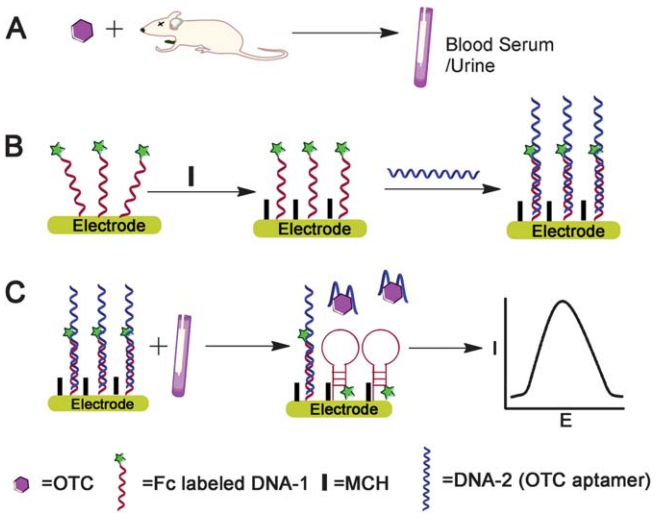
2.4 Electrochemical measurements

Test samples were incubated with the prepared functionalized gold electrode for 1.5 h. The electrode was then treated with the hybridization buffer for another 1.5 h. After washing with double-distilled water, the electrode was ready for electrochemical measurements.

Electrochemical measurements were performed on an Electrochemical Analyzer (CHI660C, CH Instruments) at room temperature. The three-electrode system consisted of the modified gold electrode as the working electrode, a saturated calomel reference electrode (SCE) and a platinum auxiliary electrode. The electrochemical technique of square wave voltammetry (SWV) was used in this study at room temperature and 200 mM PBS buffer (pH 7.4) was employed as the electrolyte for the SWV experiments. The scan rate was 50 mV s^{-1} and a 60 Hz amplitude was used in the SWV experiments. All the electrochemical measurements were repeated at least four times, and a newly prepared functionalized gold electrode was used each time.

3 Results and discussion

Scheme 1 illustrates the principles of this strategy to quantify OTC. Firstly, 5′-thiolated DNA-1 is immobilized on the surface of a gold electrode through Au–S bonds.[16–18] In the presence of DNA-2, *i.e.* the OTC aptamer, rigid dsDNA forms on the surface *via* a partial hybridization between the two oligonucleotides. The redox-active electrochemical probe Fc at the 3′-end of DNA-1 is distant from the electrode. As a result, an electrochemical signal cannot be easily obtained since the



Scheme 1 An illustration of the principles of the strategy. (A) Collection of mouse blood serum and urine as the test samples. (B) The surface of a gold electrode is functionalized with a dsDNA monolayer (formed from Fc-labeled DNA-1 and DNA-2, *i.e.* the OTC aptamer). (C) OTC in blood serum or urine is captured by DNA-2, contributing to the generation of Fc signals at 0.37 V.

electroactive Fc is far away from the electrode. When OTC is introduced, it rivals DNA-1 to bind to the OTC aptamer, leaving the DNA-1 free to self-fold into a hairpin structure. The conformational change brings the Fc probe close to the electrode, resulting in a signal-on response. Therefore, by monitoring the electrochemical response of Fc that is labeled on the 3′ end of DNA-1, the electrochemical detection of OTC can be achieved.

The competitive binding of OTC with its aptamer plays a key role in the sensing system. We have optimized the binding temperature to obtain a maximal current response. Temperatures ranging from 4 to 50 °C have been tested with a fixed concentration of OTC (500 ng ml^{-1}). As is shown in Fig. 1, the current of the SWV peak of the Fc probe increases when temperature is increased, and reaches a plateau after 37 °C, which is physiological temperature. In view of the detection of OTC in real samples, physiological temperature may be favorable. So, a binding temperature of 37 °C is adopted for the following experiments.

Next, the concentration of OTC introduced was varied to obtain quantitative detection. Fig. 2 shows the SWV responses of Fc probe under different concentrations of OTC. It can be observed that the current response is very weak if OTC is absent, suggesting that there is separation between the probe and the electrode. In the presence of OTC, the peak current increases significantly, indicating that the sensing system has worked. The current has a positive relationship with the concentration, and a linear range from 10 to 600 ng ml^{-1} can be obtained (Fig. 3). The linear regression equation is $y = 0.0044x - 0.005$ ($R^2 = 0.99$). The detection limit is determined to be 9.8 ng ml^{-1} (calculated by the function of $3\sigma/S$, where σ is the standard deviation of the blank solution and S is the slope of the linear relationship), which is much lower than the temporary ADI and MRL suggested by the WHO. The favorable sensitivity of the biosensor lays a foundation for the sensitive assay of real samples. Furthermore, because the Fc-labeled DNA-1 is tightly attached to the electrode through covalent bonds, the biosensor can also be regenerated. After being washed with 95 °C water for 30 s, the electrode can be reused to test another sample by treating the used modified electrode with DNA-2 and the stabilization buffer in turn to re-form a dsDNA-functionalized gold electrode.

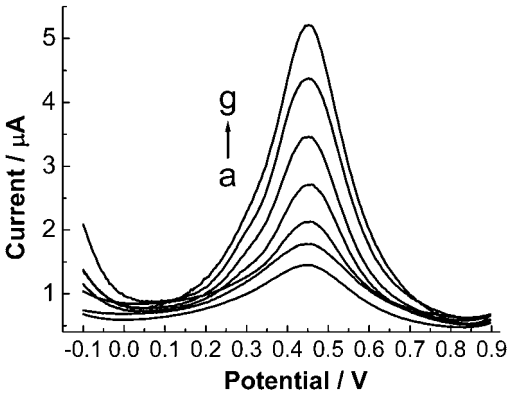


Fig. 2 Square wave voltammograms of the biosensor for the detection of various concentrations of OTC. Curves (a)–(g) represent 0, 10, 50, 100, 300, 500, and 1000 ng ml^{-1} OTC, respectively.

To study the specificity of the biosensor, we introduced some other antibiotics (penicillin and streptomycin) as controls. When the control species with a concentration equal to that of OTC are introduced, the current response is 10^4 times lower than that of OTC, suggesting that this biosensor has a superior specificity. If the concentration of the control species is increased 1000-fold, the electrochemical responses of the interferences are still very weak (Fig. 4), confirming the high specificity between OTC and its aptamer. The rational design of the immobilized DNA architecture may also make some contributions towards the specificity of the biosensor. Generally, the non-specific adsorption of interferents is unavoidable in practical tests, which is also a principal cause of false positive signals. Here, a non-specific adsorption of interferents onto the modified electrode may also occur. However, the non-specific adsorption of interferents is more likely to involve non-competitive binding, in which the rigid dsDNA structure is

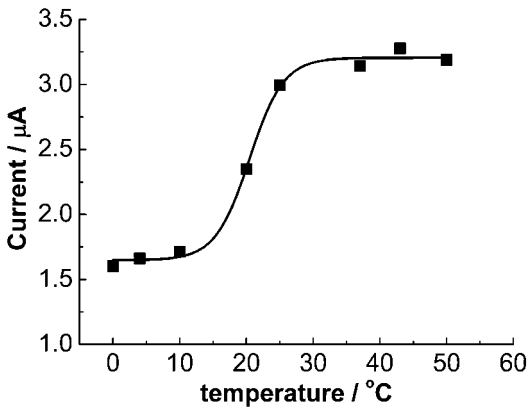


Fig. 1 Plots of peak current from SWV measurements for 500 ng ml^{-1} OTC *vs.* temperature.

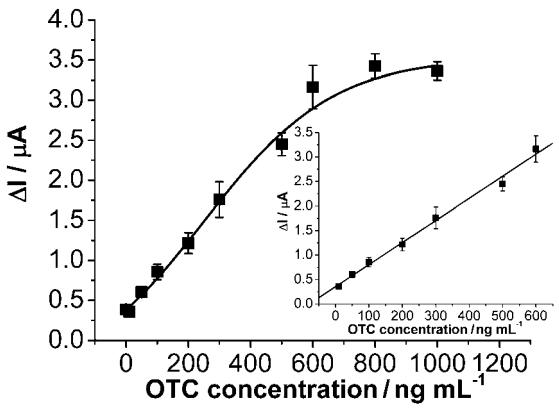


Fig. 3 The calibration curve of the SWV peak currents against the concentration of OTC. The dots are fitted with a sigmoidal function. The inset shows a linear relationship between the peak currents and the concentrations of OTC from 10 to 600 ng ml^{-1}.

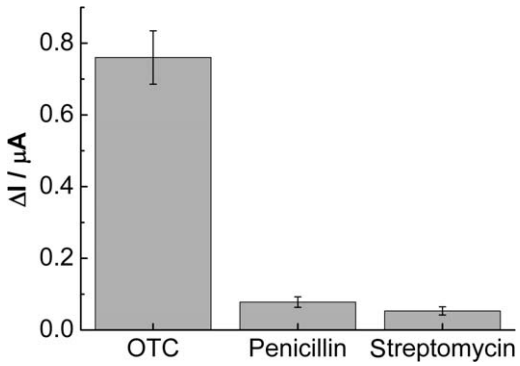


Fig. 4 Peak currents from SWV measurements for 100 ng ml^{-1} OTC, 100 μg ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

undamaged. Therefore, the position of the Fc probe may not change and the non-specific adsorption may not contribute to false positive signals. On this basis, it is also expected that the superior specificity of the aptasensor may meet the demand for use in real samples.

We then used the biosensor to quantify OTC in mouse blood serum and urine. 10 μg g^{-1} OTC was orally administrated, and the control group was given only the solvent. Blood serum and urine were collected 3.5 h after dosing, and used directly as the test samples without any pretreatment. The measurements were independently repeated four times to guarantee the repeatability of the method. Results show that the concentration of OTC is determined to be 1.2 ± 0.12 μg ml^{-1} in blood serum, and 0.5 ± 0.07 μg ml^{-1} in urine (Table 1). In the case of the control group, 0 ± 0.03 μg ml^{-1} and 0 ± 0.02 μg ml^{-1} for blood serum and urine are determined, respectively, suggesting an excellent anti-interference capability. A traditional HPLC test is also performed to confirm the results. In the HPLC test, the concentration of OTC is determined to be 1.3 ± 0.11 μg ml^{-1} in blood serum, and 0.5 ± 0.06 μg ml^{-1} in urine. The results of the HPLC test and the detection of OTC using our biosensor seem to be almost identical. Therefore, it follows that our biosensing system works well and can be adopted for the detection of OTC in real samples.

It should be mentioned that serum is not an ideal sample for some detection systems. Pre-purification is usually required due to its high viscosity (relative viscosity of ~1.6) and high concentrations of salts (~150 mM), proteins (serum total protein of 60–80 g l^{-1}), and a variety of other molecules. Here, we are able to detect OTC levels in serum directly due to the powerful anti-interference capability of the biosensor. Although HPLC, the most commonly used method, also works well for the detection of OTC compared with our method in terms of sensitivity, complex processes are required. It is not favorable for further batch measurements and miniaturization. Therefore, the fabricated biosensor in this work might have great potential applications in the future.

4 Conclusions

In conclusion, we have successfully developed a promising aptasensor for the detection of OTC, a widely used antibiotic. Its extensive use has increased the risk of chronic drug abuse. In our strategy, the specific recognition of OTC using an aptamer is skillfully integrated with electrochemical detection techniques. The architecture is simple, easily-constructed, and has the ability to be regenerated. What is more, by differentiating specific and non-specific binding, the strategy allows the remarkable depression of false positive signals, facilitating the application in the detection of OTC in real samples. Experimental results show that the biosensor has favorable sensitivity, selectivity, and anti-interference capabilities. On this basis, we are able to detect OTC in mouse blood serum and urine directly. Further development of this method may have applications in food security as well as in clinical diagnosis and the study of pharmacokinetics, since the extensive abuse of OTC may cause gastrointestinal and photosensitive allergic reactions. Along with the development of aptamer technology, alternative uses of this method for the electrochemical detection of other antibiotics and/or drug compounds may also be achieved in the future.

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Table 1 The concentration of OTC in real samples measured using the biosensor and the HPLC method, respectively

	Blood serum			Urine		
Sample no.	Experimental group (aptasensor) (μg mL^{-1})	Experimental group (HPLC) (μg mL^{-1})	Control group (aptasensor) (μg mL^{-1})	Experimental group (aptasensor) (μg mL^{-1})	Experimental group (HPLC) (μg mL^{-1})	Control group (aptasensor) (μg mL^{-1})
1	1.15	1.24	0.008	0.49	0.52	−0.02
2	1.31	1.31	0.02	0.57	0.60	−0.001
3	1.09	1.19	−0.026	0.51	0.56	0.009
4	1.26	1.41	0.004	0.44	0.46	0.003
Average	1.2 ± 0.12	1.3 ± 0.11	0 ± 0.03	0.5 ± 0.07	0.5 ± 0.1	0 ± 0.02

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