Fast determination of the tetracyclines in milk samples by the aptamer biosensor

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A novel aptamer biosensor for fast tetracyclines determination was established and illustrated in this paper. The tetracyclines aptamer as a specific affinity molecule was immobilized on the surface of the glassy carbon (GC) electrodes. It can specifically bind tetracyclines quickly in milk and other samples without sample treatment. Subsequently, the electrochemical signals are produced and measured accurately. The linear relationship between the currents and the tetracyclines concentration is 0.1–100ng ml^{-1}. The sensitivity limit of the device is 1ng ml^{-1}. The detection time is 5 min.

Introduction

Aptamers are specific nucleic acid fragments, which can bind to a wide range of non-nucleic acid targets with high affinity and specificity.[1] They are identified and selected through an *in vitro* process, which is termed as systematic evolution of ligands by exponential enrichment (SELEX).[2,3]

On the other hand, tetracyclines are a group of antibiotics, such as tetracycline and terramycin. They are widely used for human and animal disease treatments.[4–6] However, tetracyclines abuse can affect consumer health. In order to prevent harm, a novel method for fast tetracyclines detection is eagerly demanded.

At present, the methods for tetracyclines determination are enzyme linked immunosorbent assay (ELISA), thin layer chromatography, capillary electrophoresis, and high performance liquid chromatography (HPLC). However, most require large instruments and professional operators, and they are expensive techniques.

Recently, it has been demonstrated that aptamer biosensors possess many advantages of specificity, affinity, stability, and high sensitivity.[7–14] These usually change the conformation upon aptamer binding, which results in either emitting a fluorescent signal based on the fluorescent label, triggers an electrochemical sensor or leads to a change of colour.

Yoshida *et al.* constructed an aptamer biosensor. It was composed of an enzyme inhibiting aptamer and a target molecule binding aptamer. The inhibiting aptamer folded into the G-quartet structure to inhibit the enzyme activity. While, the target molecule binding aptamer inserted into the inhibiting aptamer to change the G-quartet structure. The analyte was detected by the enzyme activity analysis.[15,16] Later on, Mir and colleagues developed a chronoamperometric beacon biosensor based on a ferrocene labelled thiol-aptamer. The system was demonstrated with impedance spectroscopy and chronoamperometry measurements. The detection limit was 30fM with the impedance spectroscopy.[17] Kenzo and co-workers investigated an aptamer-based lable-free sensor for immunoglobulin E (IgE) detection. It employed the carbon nanotube field-effect transistors (CNTFETs). When the immobilized aptamers on CNT channels reacted with IgE at various concentrations, the current was decreased sharply. So the concentration of IgE was determined, and the sensitivity was 1.9-10^{-9}M.[18]

In the present research, the tetracyclines aptamer was coupled with the amino group modified GC electrode *via* the intermediates EDC and NHS. During the reaction, the phosphate group of the oligonucleotide aptamer formed the phosphate amine ester

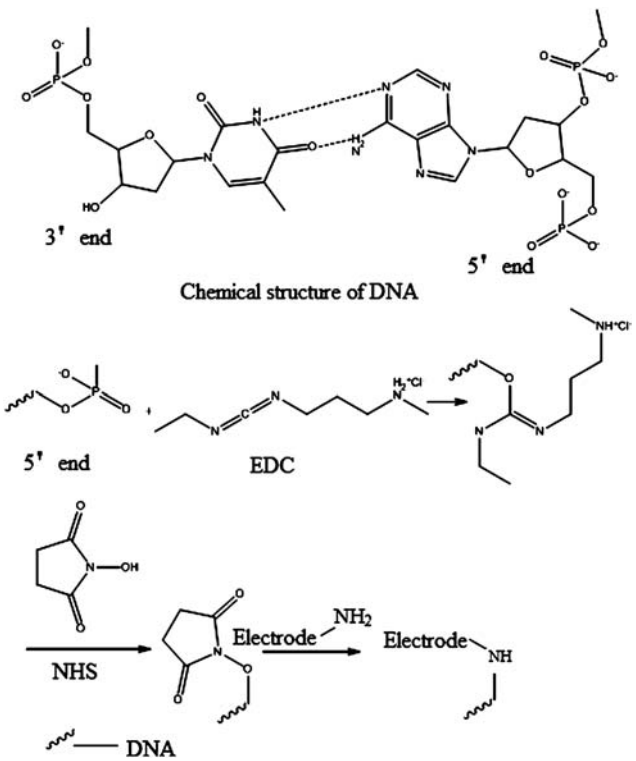

Fig. 1 Schematic illustration of the aptamer immobilization on an amino group modified GC electrode.

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and linked to the electrode (Fig. 1). Thus, the aptamer immobilized electrode can specifically pick up the tetracyclines in milk samples without sample treatment. Thereafter, the electrochemical signal can be produced as a result of the tetracyclines concentration changes, and can be detected quickly. This novel aptamer biosensor also has advantages of being label free, specific, convenient to carry, inexpensive and sensitive.

Experimental

Materials and equipment

BSA, 3-aminopropyltriethoxysilane, $K_3Fe(CN)_6$, tetracycline, terramycin, penicillin, EDC and NHS were purchased from Beijing Dingguo biology company.

The electrochemical experiments were undertaken on a LK2005A electrochemical workstation (Tianjin Lanlike company) and a conventional three electrodes system. The working electrode was a tetracyclines aptamer immobilized GC electrode. A platinum electrode was applied as the counter electrode, and an Ag/AgCl electrode acted as the reference electrode.

The working electrode preparation

The GC electrode was first polished with Al_2O_3 and then cleaned thoroughly. After it was dried with a stream of nitrogen gas, the electrode was electrochemically oxidized by cyclic voltammetry at potential of −0.2–1.8V. Subsequently, it was immersed into the lithium aluminium tetrahydride ether solution at room temperature for 1 h, so that the surface of the electrode was modified with hydroxyl groups. Next, the amino group attachment on the electrode was undertaken by reacting it with a freshly made solution of 3-aminopropyltriethoxysilane: HCl: distilled water (1 : 15 : 10) at 53 °C for 3 h. Henceforth, the electrode was soaked in 0.05M EDC and 0.03M NHS water solution to activate the amino-terminated surface at 50 °C for 90 min.

Finally, the aptamer immobilization was achieved by adding 10μL of 25ng μL^{-1} tetracyclines aptamer on the surface of the modified GC electrode under ultraviolet radiation for 1 h. The electrode was then rinsed with distilled water thoroughly and blocked with 5μL of 1%BSA for 2 h at room temperature. It was further applied as the working electrode. Meanwhile, the GC electrode used as a negative control was also treated as above except the aptamer immobilization.

Optimization of the aptamer immobilization concentration

The tetracyclines aptamer was made by SELEX method. The tetracycline was coated on a 96-well ELISA plate. The ssDNA was incubated with the tetracycline coated wells at 37 °C for 40 min. Subsequently the unbound ssDNA was washed out. The bound ssDNA was eluted with phenol-chloroform, and isolated with alcohol.

The GC electrode was first immobilized with 10μL of 4 concentrations of the aptamer (40ng μL^{-1}, 35ng μL^{-1}, 30ng μL^{-1} and 25ng μL^{-1}) separately. Then they were washed with 30μL of distilled water. Afterwards, the unbound aptamer in the washing solution was examined by the DNA gel electrophoresis.

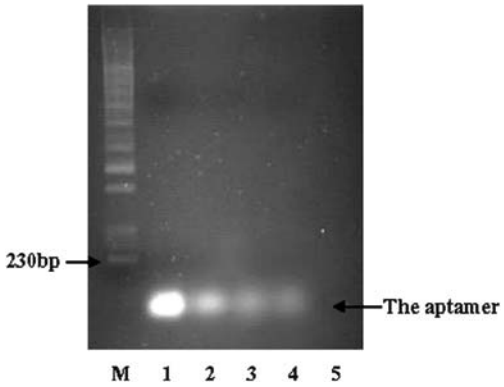

Fig. 2 The aptamer DNA gel electrophoresis. (M) The DNA marker. (1) 25ng μL^{-1} aptamer. (2) washing solution from the 40ng μL^{-1} aptamer immobilization. (3) washing solution from the 35ng μL^{-1} aptamer immobilization. (4) washing solution from the 30ng μL^{-1} aptamer immobilization. (5) washing solution from the 25ng μL^{-1} aptamer immobilization.

The antibiotics analysis with the aptamer biosensor by electrochemical methods

The electrochemical measurements with the aptamer biosensor were carried out in a single compartment of a three electrode cell, which initially contained 10mL of 0.15mM $K_3Fe(CN)_6$ solution. A series of concentrations of the antibiotics were prepared in either PBS or milk. They were additionally applied to the cell. Next, the cyclic voltammograms were recorded, and the concentrations *versus* the peak current were plotted as the standard curve. The antibiotics in milk samples were determined in the same manner, and the concentrations were calculated according to the standard curve.

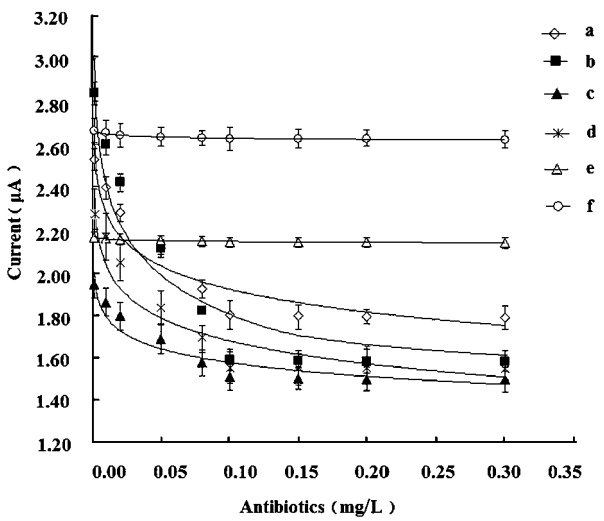

Fig. 3 The plot of the antibiotics concentration *versus* the current. (a) The tetracycline PBS determination. (b) The tetracycline milk determination. (c) The terramycin PBS determination. (d) The terramycin milk determination. (e) The penicillin PBS determination. (f) Negative control.

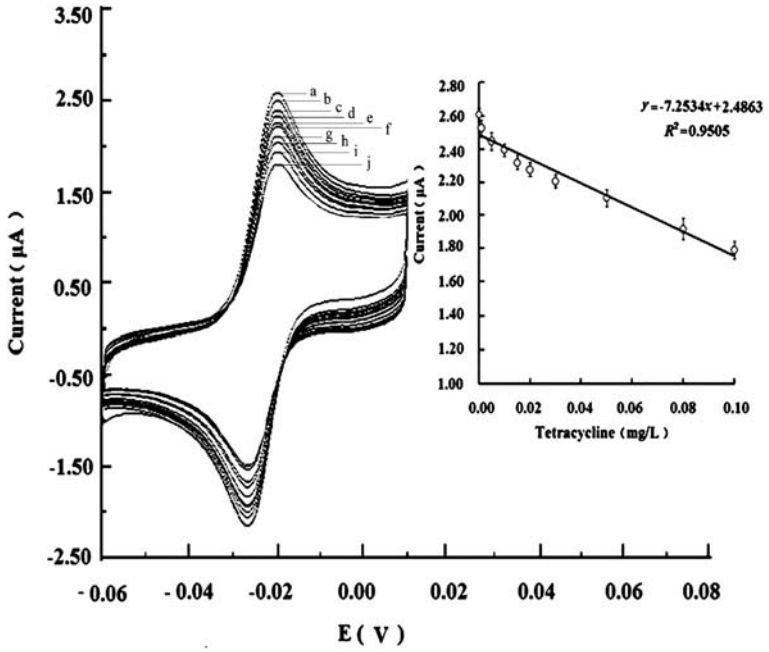

Fig. 4 Cyclic voltammograms of the tetracycline PBS determination. Therein the tetracycline concentrations are (a) 0mg L^{-1}, (b) 0.001 mg L^{-1}, (c) 0.005 mg L^{-1}, (d) 0.01 mg L^{-1}, (e) 0.015 mg L^{-1}, (f) 0.02 mg L^{-1}, (g) 0.03 mg L^{-1}, (h) 0.05 mg L^{-1}, (i) 0.08 mg L^{-1}, (j) 0.1 mg L^{-1}. The inset is the plot of the tetracycline concentration *versus* the peak current at −0.02V.

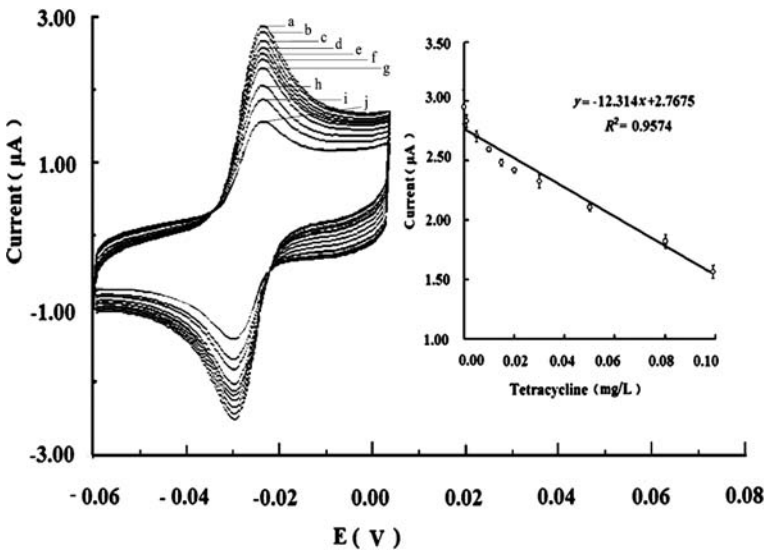

Fig. 5 Cyclic voltammograms of the tetracycline milk determination. Therein the tetracycline concentrations are (a) 0mg L^{-1}, (b) 0.001 mg L^{-1}, (c) 0.005 mg L^{-1}, (d) 0.01 mg L^{-1}, (e) 0.015 mg L^{-1}, (f) 0.02 mg L^{-1}, (g) 0.03 mg L^{-1}, (h) 0.05 mg L^{-1}, (i) 0.08 mg L^{-1}, (j) 0.1 mg L^{-1}. The inset is the plot of the tetracycline concentration *versus* the peak current at −0.02V.

Results and discussion

Optimization of the aptamer immobilization concentration

It can be seen in Fig. 2 that M is the DNA marker, line 1 is the 25ng μL^{-1} aptamer without immobilization, and it plays a positive control part. Line 2 is the washing solution from the 40ng μL^{-1} aptamer immobilization, line 3 is the washing solution from the 35ng μL^{-1} aptamer immobilization, and line 4 is the washing solution from the 30ng μL^{-1} aptamer immobilization. They all appear in the aptamer band. Nevertheless, line 5 is the washing solution from the 25ng μL^{-1} aptamer immobilization, and there is no clear band. This clarifies that all 10μL of 25ng μL^{-1} aptamer is immobilized on the GC electrode. Therefore, 25ng μL^{-1} is the maximum immobilization concentration.

The aptamer biosensor properties study using the electrochemical method

The aptamer immobilized electrodes were employed to analyze the antibiotics in 0.15 mM $K_3Fe(CN)_6$ solution with the cyclic

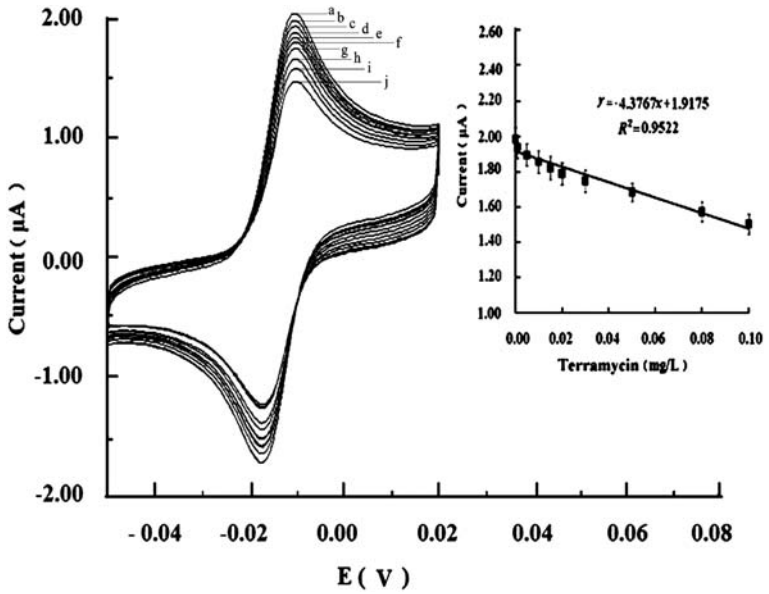

Fig. 6 Cyclic voltammograms of the terramycin PBS determination. Therein the terramycin concentrations are (a) 0mg L^{-1}, (b) 0.001 mg L^{-1}, (c) 0.005 mg L^{-1}, (d) 0.01 mg L^{-1}, (e) 0.015 mg L^{-1}, (f) 0.02 mg L^{-1}, (g) 0.03 mg L^{-1}, (h) 0.05 mg L^{-1}, (i) 0.08 mg L^{-1}, (j) 0.1 mg L^{-1}. The inset is the plot of the terramycin concentration *versus* the peak current at −0.02V.

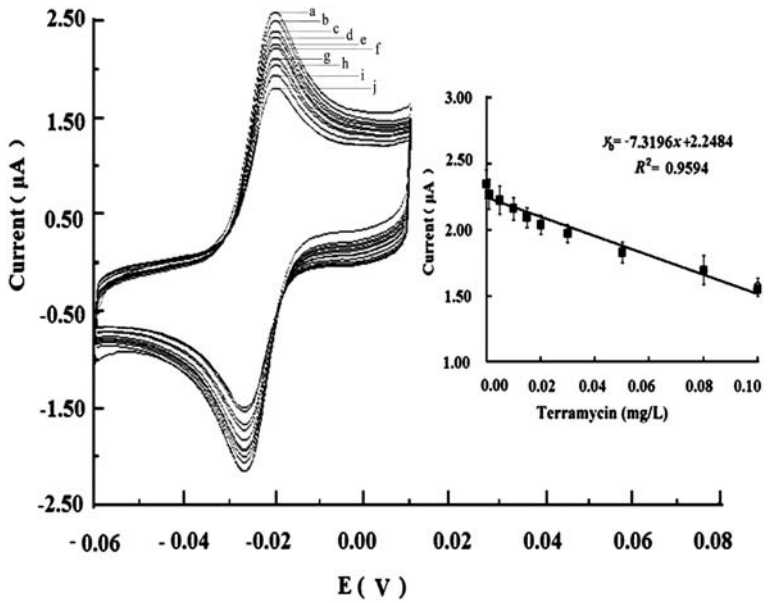

Fig. 7 Cyclic voltammograms of the terramycin milk determination. Therein the terramycin concentrations are (a) 0mg L^{-1}, (b) 0.001 mg L^{-1}, (c) 0.005 mg L^{-1}, (d) 0.01 mg L^{-1}, (e) 0.015 mg L^{-1}, (f) 0.02 mg L^{-1}, (g) 0.03 mg L^{-1}, (h) 0.05 mg L^{-1}, (i) 0.08 mg L^{-1}, (j) 0.1 mg L^{-1}. The inset is the plot of the terramycin concentration *versus* the peak current at −0.02V.

voltammetry. Thereby the $Fe_3(CN)_6^{3-}/Fe_2(CN)_6^{4-}$ redox couple was taking place on the surface of the working electrode. When the solution contains the tetracyclines, the aptamer on the electrode will bind the tetracyclines. Further the redox system is interrupted and the peak current is decreased. The antibiotics were made either in PBS or milk. The antibiotics concentration *versus* the peak current was plotted (Fig. 3). Fig. 3a is the tetracycline PBS determination using the aptamer biosensor. Fig. 3b is the tetracycline milk determination using the aptamer biosensor. Fig. 3c is the terramycin PBS determination using the

Table 1 Determination of tetracyclines in the milk samples

Tetracyclines	Real concentration ng L^{-1}	Measured concentration ng L^{-1}	CV (%)
Tetracycline	45.0	43.5	4.9
Tetracycline	65.0	67.0	4.2
Tetracycline	85.0	88.5	5.6
Terramycin	45.0	42.5	8.3
Terramycin	65.0	67.8	6.2
Terramycin	85.0	82.5	4.3

aptamer biosensor. Fig. 3d is the terramycin milk determination using the aptamer biosensor. Fig. 3e is the penicillin PBS determination using the aptamer biosensor. Fig. 3f is the tetracycline PBS determination using the non aptamer immobilized GC electrode.

Results from Fig. 3a–d illustrate that the aptamer biosensor specifically binds to tetracyclines and the concentration can be analyzed with the electrochemical method, since the current is decreased with increasing concentration of the tetracycline and the terramycin. When the concentration is lower than 100μg L^{-1}, the current decreases rapidly. However, when the concentration is higher than 100μg L^{-1}, the current remains at a constant level. In spite of that, there is no significant current change in Fig. 3e and 3f. This shows that the aptamer does not recognize the penicillin, but has specificity to tetracyclines. As well as the non aptamer immobilized GC electrode does not bind to the tetracyclines.

Affinity constant calculation

Fig. 2 reveals that about 250ng (9.6×10^{-12} mols) of aptamer is immobilized on the electrode. The electrochemical measurement cell contains 10ml of solution with different concentrations of the tetracyclines. The saturated binding concentration is 100μg L^{-1} (Fig. 3), 10ml measurement solution contains 2.1×10^{-9} mols of tetracyclines. So the affinity constant is 218, *i.e.*, 1mols aptamer can bind 218 mols tetracyclines.

The tetracyclines determination using the aptamer biosensor

A series of the tetracycline concentrations was measured using the aptamer biosensor with cyclic voltammetry. The tetracycline concentrations in either PBS (Fig. 4) or milk (Fig. 5) *versus* the peak current at −0.02V are plotted as the standard curve. The current is linear with the tetracycline concentration, when the concentration is in the range of 0.1–100μg L^{-1}, and the detection limit is 1μg L^{-1}.

Similarly, the terramycin determination using the aptamer biosensor was investigated with cyclic voltammetry. A series of terramycin concentrations in either PBS (Fig. 6) or milk (Fig. 7) *versus* the peak current at −0.02V are plotted as the standard curve. The linear relationship between the terramycin concentration and the current is 0.1–100μg L^{-1}, and the detection limit is 1μg L^{-1}, which is the measurement data, *i.e.*, when the concentration is 1μg L^{-1}, there will be a current decrease.

Evaluation of the aptamer biosensor

Six concentrations of the tetracyclines in the milk samples were accurately prepared and confirmed. They were detected using the aptamer biosensor with the same system as above and the results were obtained according to the standard curve from Fig. 4–7. Also the Coefficient of Variation (CV) was calculated. They are all less than 10% (Table 1). This explicates that the biosensor produces precise results.

Conclusions

The tetracyclines aptamer biosensor was developed. The device can detect tetracyclines in the milk samples without sample treatment. It is simple, inexpensive, rapid, precise, sensitive and label free. The linear relationship between the tetracyclines concentration and the current is 0.1–100ng ml^{-1}, and a detection limit of 1 ng ml^{-1} is achieved. In addition, the device specificity was analyzed with penicillin. The results are shown in Fig. 3e and 3f. There is no significant interference from penicillin. The non aptamer immobilized GC electrode does not bind to the tetracyclines.

Acknowledgements

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