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Short Communication

Ultra-Sensitive Aptasensor Based on IL and Fe_3O_4 Nanoparticles for Tetracycline Detection

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An aptasensor for detecting tetracycline residues in milk was fabricated by depositing an ionic liquid (IL) and Fe_3O_4 magnetic nanoparticles onto a screen-printed electrode (SPE). IL has great conductivity and a large electrochemical window, making it a new green medium for such devices. Fe_3O_4 enhances electrochemical signals and forms films on surfaces well. As the composite mediators, these materials not only accelerated electron transfer but also improved the response speed and precision of the aptasensor. Herein, the synergistic effect of IL and Fe_3O_4 was exploited to construct a sensing interface for amplifying signals. The proposed aptasensor was capable of detecting tetracycline as demonstrated here. The detection range of the aptasensor was 1.0 nM to 1.0×10^7 nM with a detection limit of 1.0 nM, which is much lower than the maximum residue limit allowed by international regulations (224 nM). The aptasensor demonstrated good selectivity, reproducibility, and stability. Additionally, the sensor can be configured for the detection of other antibiotics.

Keywords: Ionic liquid; Fe_3O_4; Aptasensor; Tetracycline

1. INTRODUCTION

Antibiotics are commonly used in human and veterinary medicine and have attracted increasing attention as emerging environmental contaminants[1]. In addition to their ecotoxicity[2,3], antibiotics in the environment may impose selective pressure on bacteria, triggering the development of antibiotic resistance in bacterial populations, thus creating a threat to public health. Tetracycline-based antibiotics (TET), such as tetracycline (TC) and oxytetracycline (OTC), are recalcitrant, broad-spectrum antimicrobial agents widely present in wastewater and natural water bodies[4,5]. TET

(Figure 1) has the molecular formula $C_{22}H_{24}N_2O_8$ and a molecular weight of 444.4346. TET is a class of broad-spectrum antibiotics that is produced or semisynthesized by the *Actinomyces* genus of bacteria, and includes tetracycline, chlortetracycline, and oxytetracycline. TETs are widely used in the poultry industry, not only for preventing and treating animal diseases but also for promoting growth[6].

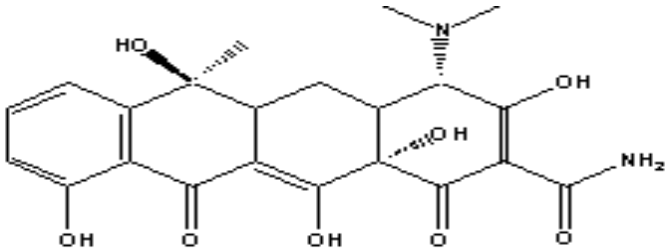

Figure 1. The molecular structure of tetracycline

Over the past few decades, many traditional analytical methods have been used to detect TET, including high-performance liquid chromatography (HPLC)[7-9], capillary electrophoresis (CE)[10,11], fluorescence (FL)[12,13] and mass spectrometric detection (MS)[14,15]. These traditional analytical methods are highly sensitive and specific; however, due to the expense of the equipment and complex sample pretreatment procedures, these methods are neither suitable for rapid detection nor widely used. Kurittu [16] used a microbial inhibition method to detect the TET residues, but this method lacked specificity and sensitivity. Therefore, it is necessary to establish a simple, sensitive and selective detection method for TET residues.

Over the last few years, some rapid detection methods of TET by electrochemical methods have been developed, such as enzyme-based sensors[17], immunosensors[18], and aptasensors[19]. An aptasensor is a type of biosensor in which an aptamer is used as a biological activity probe. An aptamer is a nucleic acid ligand selected from an external random sequence library that can be naturally folded into different three-dimensional structures and combined with specific capabilities to bio-surfaces[20-23]. Furthermore, an aptamer can be identified by an iterative approach called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) to capture a specific target[21]. Aptamers have several advantages over antibodies as detection ligands, such as high stability, more simplicity, easy modification, target versatility, low cost and synthetic manufacture. Electrochemical aptasensors used for TET detection in food safety analysis have been reported[19,24]. To detect TET, Kim [24] developed an electrochemical aptasensor based on a screen-printed gold electrode using square-wave voltammetry (SWV); however, the proposed aptasensor suffered the drawbacks of low sensitivity, poor stability and poor reproducibility. Hence, current research has focused on finding ways to modify aptasensors with materials that improve detection sensitivity.

An electrochemical window[25] acting as a new class of green media[26] has been developed in recent years. Ionic liquids can replace conventional organic solvents or acid-base solvents, not only acting as a new medium[27] with a chemical and separation reaction but also showing potential as a new magnetic material[28], a nano- or micro-structure functional material[29], a lubrication

material[30] or an aerospace propellant[31]. Because ionic liquids have the characteristics of high conductivity, less volatility, and inflammability, the potential window of electrochemical stability is much larger than that of other aqueous electrolyte solutions. As a result, they have been widely used in electrochemical biosensor preparation[32,33] in recent years.

Fe_3O_4 magnetic nanoparticles can enhance electrochemical signals[34] and have been widely used in electrochemical sensors. Also, Fe_3O_4 magnetic nanoparticles can form a thin film on the electrode surface, increasing the electrode area so that redox-active substances can be transported to the electrode surface[35,36]. An electrode modified with Fe_3O_4 magnetic nanoparticles has a greatly improved ability to bind with a biomolecule and to function as a sensor[37-39]. Compared to the traditional electrodes used in electrochemical sensing, a screen-printed electrode (SPE) provides the possibility of the mass production of reproducible electrochemical sensing devices due to their inexpensive, simple, rapid, and versatile characteristics [40,41]. To the best of our knowledge, an aptasensor with ionic liquid (IL) and an SPE modified with Fe_3O_4 magnetic nanoparticles used for detecting tetracycline residues in milk has not been previously reported.

In this work, a novel label-free electrochemical aptasensor based on IL and Fe_3O_4 on a microarray electrode was fabricated. Herein, IL was the material for the first layer of the modified electrode designed to act as a "molecular wire" to facilitate electron transfer. Fe_3O_4 served as the second layer on the electrode. TET was captured by the aptamer immobilized on the Fe_3O_4/IL/SPE, and the step change of the modified electrode was investigated by cyclic voltammetry (CV). Our experiments show a wide target recognition range of this ultra-sensitive aptamer, an inherent advantage for this aptasensor to be applied to other analyses[6]. This aptasensor was successfully used in the detection of tetracycline antibiotic residues in real milk samples.

2. EXPERIMENTAL

2.1 Apparatus

Electrochemical measurements were carried out with a CHI660D electrochemical workstation (Chenhua Co., Shanghai, China). The detection electrode was a TE100 screen-printed electrode (SPE) purchased from Zensor R&D Co. (Taiwan). Solution pH value was measured with an FE20 Mettler-Toledo pH meter (Switzerland). All electrochemical experiments were carried out at room temperature (RT).

2.2 Reagents and materials

The anti-TET oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and the following sequences were designed by Niazi[34] and Zhou[23]: 5'-NH_2-(CH_2)-CGT ACG GAA TTC GCT AGCCCC CCG GCA GGC CAC GGC TTG GGT TGGTCC CAC TGC GCG TGG ATC CGA GCT CCACGT G -3' and probe aptamer 5'-GCA TGC CTTAAG CGA TCG GGG GGC CGT CCG GTG CCGAAC CCA ACC AGG GTG ACG CGC ACC TAGGCT CGA GGT GCA C-6 FAM (FITC)-3'. Chloroauric acid ($HAuCl_4$) and

ethanol were acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chitosan (CS) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Phosphate buffer solution (PBS, pH = 7.4) was prepared from 0.1 g KH_2PO_4, 1.45 g $Na_2HPO_4 \cdot 12H_2O$, 0.1 g KCl and 4 g NaCl in 500 mL ultrapure water with its pH adjusted by NaOH and HCl solutions. The N-octylpyridinium hexafluorophosphate (purity > 99%) was purchased from Shanghai Chengjie Chemical Co., LTD. The other chemicals were analytical reagent grade and used without further purification. Ultrapure water (18.2 MΩ•cm) was prepared by a PALL system (PALL Corporation, USA).

2.3 Preparation of Fe_3O_4 and IL solution

Before the experiment, 2% (2 g/100 mL) chitosan solution was prepared by dissolving 2.0 g chitosan flakes into 100 mL 1.0% acetic acid and stirring for 3 h. Then, 1 mg Fe_3O_4 powder was added into the 4 mL 2% CS solution, and the solution was oscillated for 6 h to prepare the Fe_3O_4 solution. Similarly, the 2 mg/mL IL was prepared by adding 2 mg N-octylpyridinium hexafluorophosphate powder into 4 mL ethanol and sonicating for 6 h. These solutions were stored in a 4 ℃ refrigerator.

2.4 Fabrication of the aptasensor

Prior to fabrication of the aptasensor, the electrodes were oscillated sonically in 1 mM NaOH solution, 1 mM HCl, and ethanol for 5 min, then washed with ultrapure water and dried under nitrogen. Next, the electrodes were electrochemically cleaned in 0.5 M H_2SO_4 by potential scanning between -0.5 V and +1.5 V for 5 min, then washing with ultrapure water and drying under nitrogen.

After the electrodes were cleaned and activated, 6 μL of the 0.5 mg/mL IL was pipetted onto the surface of the SPE. Then, the modified electrode (IL/SPE) was dried at RT before 6 μL of the Fe_3O_4 was cast onto the pre-treated substrate and dried over 2 h at RT to get Fe_3O_4/IL/SPE. Then, 20 μL of the 5 mM aptamer was dropped onto the surface of the pre-treated electrode for one night at RT. After that, 20 μL TET was immobilized on the dried SPE for 10 min at RT and the electrode was washed with ultrapure water. Then, 20 μL of variable concentrations of TET was dropped onto the surface of the electrode for 40 min at RT and then washed with ultrapure water. Finally, the fabricated electrode TET/Apt/Fe_3O_4/IL/SPE was stored at 4 °C when not in use. The obtained electrode was used as the aptasensor in this work, and the stepwise assembly of the proposed aptasensor is shown in Fig. 2.

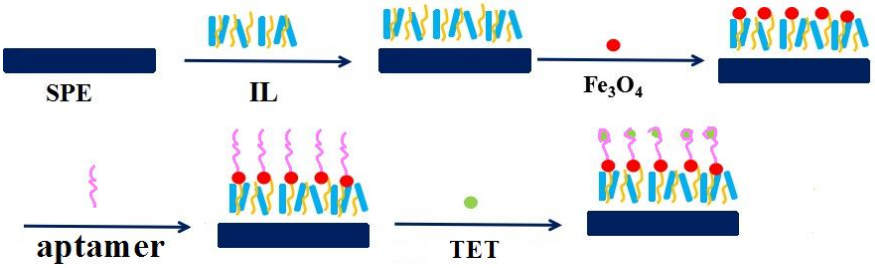

Figure 2. Combination process of aptasensor

2.5 Electrochemical measurements

The electrochemical characteristics of the modified electrode were characterized by CV in 5 mM $[Fe(CN)_6]^{3-/4-}$. Electrochemical measurements were performed in a traditional electrochemical cell. By dropping 20 μL of various concentrations of the target TET on the Apt/Fe_3O_4/IL/SPE at RT for 40 min anti-TET/TET complexes were generated. By washing carefully with ultrapure water, the unbound TET was removed. Then, CV for the detection of TET was performed to characterize the electrode. The CV measurements were performed in the potential range from -0.2 V to +0.6 V with pulse amplitude of 50 mV.

3. RESULTS AND DISCUSSION

3.1 Fabrication mechanism of the sensor

The CV data of the layer-by-layer modified electrodes are presented in Fig. 3. The bare SPE had an obvious redox peak (Fig. 3(a)). After the IL (Fig. 3(d)) was immobilized on the bare SPE, a larger current response was detected. This result was consistent with that found in the literature[42]. The reason for this response might be that IL can accelerate the charge transfer between the liquid and the electrode and enhance the peak of the current. With 6 μl Fe_3O_4 added onto the IL-modified electrode, the Fe_3O_4/IL/SPE exhibited a much higher current as shown in Fig. 3(e). This suggests that the Fe_3O_4 increases the charge exchange between the electrode and the solution. After the 5 μL 5 mM aptamer was added to the surface of the Fe_3O_4/IL/SPE, the peak current decreased in sequence (Fig. 3(c)). This phenomenon suggests that the aptamer significantly reduced the effective area and active sites for electron transfer. With the fabricated SPE remaining in the sample solution for some time, the current peak decreased further (Fig. 3(b)) presumably because the aptamer and TET specifically reacted and formed non-conductive composites.

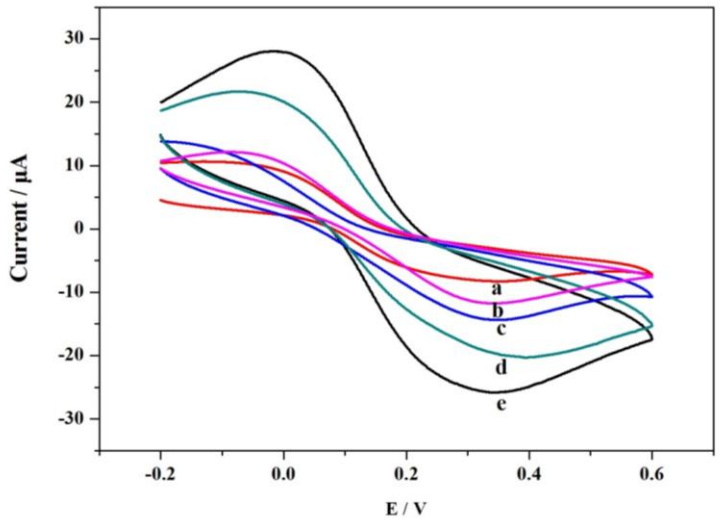

Figure 3. The CV data of layer-by-layer modified electrodes: (a) Bare SPE, (b) TET/Apt / Fe_3O_4/ IL/ SPE, (c)Apt/Fe_3O_4/IL/SPE, (d) IL/SPE, (e) Fe_3O_4/IL/ SPE

3.2 Calibration curve

The calibration plots for TET detection with the prepared aptasensor under optimal experimental conditions are shown in Fig. 4. These results are similar to those found in the literature[43]. A gradual increase in current was observed with increasing TET concentration, and the corresponding calibration curve (Fig. 5) exhibited a good linearity. These results were also similar to those found in other published reports[44]. The changes of oxidation peak current response (DI) of the aptasensor were found to be linearly proportional to the TET concentration in the range from 1×10^{-2} M to 1×10^{-9} M. The linear slope was 0.8881 and the correlation coefficient was 0.9687, which was comparable to our previous studies[45].

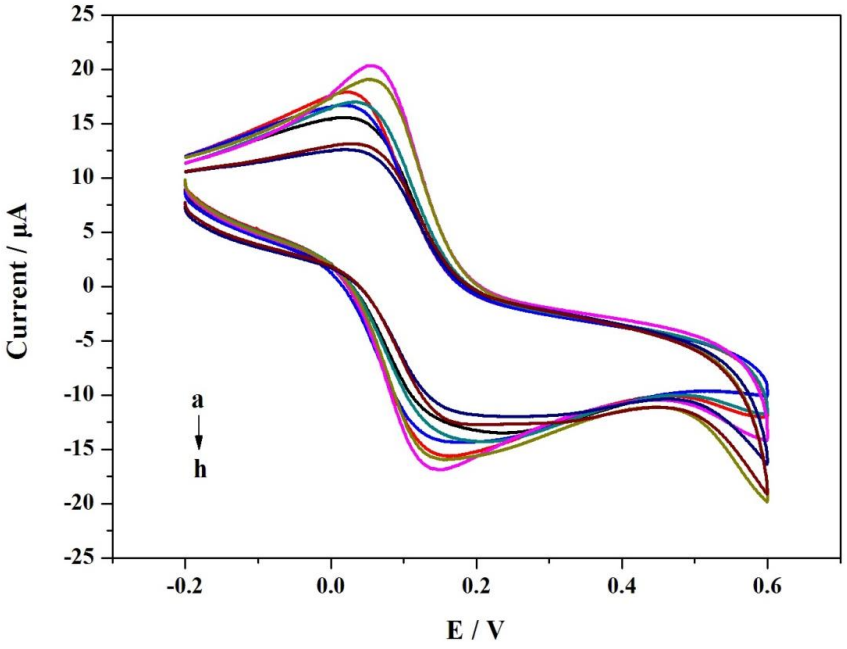

Figure 4. CV data charts of aptasensor to concentrations of TET (a-h: 1×10^{-2} M, 1×10^{-3} M, 1×10^{-4} M, 1×10^{-5} M, 1×10^{-6} M, 1×10^{-8} M, 1×10^{-9} M)

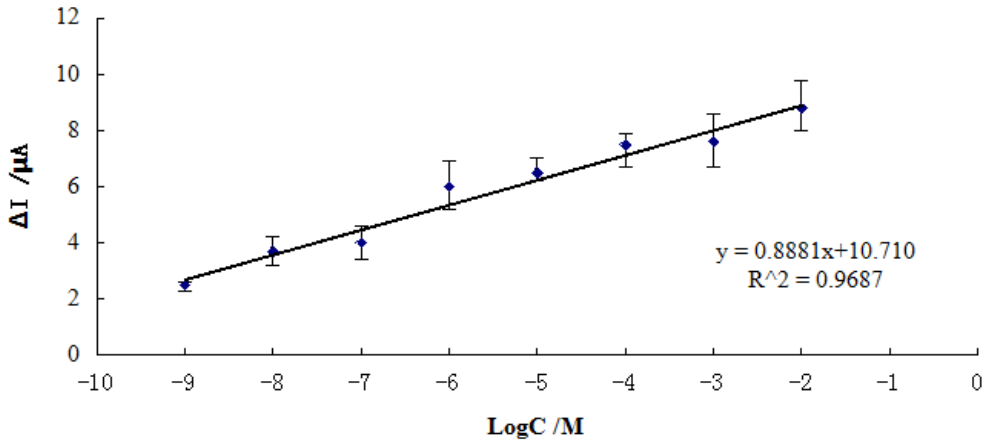

Figure 5. Standard curve of aptasensor for TET

Table 1. Comparisons of the linear range and detection limit of different electrodes in the detection of TET

Electrode	Linear range (μmol•L^{-1})	Detection limit (μmol•L^{-1})	References
Pt foil	0.045–22.5	0.009	[46]
Pt disk	9×10^{-5}–9×10^{-3}	4.5×10^{-6}	[47]
GME[a]	2.25–22.5, 22.5–225	0.23	[48]
MWCNT-GCE[b]	2.5–100	0.12	[49]
mvRuO/RuCN[c]-GCE	–	0.23	[50]
Ni-GCCME[d]	5.6–180	0.06	[51]
Fe_3O_4/IL	1.0×10^{-3}–1.0×10^{4}	0.001	This work

a. GME: gold microelectrode.
b. MWCNT-GCE: multi-walled carbon nanotubes-glassy carbon electrode.
c. mvRuO/RuCN: mixed-valence ruthenium oxide/ruthenium cyanide.
d. Ni-GCCME: nickel-glassy carbon chemically modified electrode.

3.3 Determination of tetracycline in real samples

Since the proposed aptasensor showed good selectivity toward TET, it was worth exploring an analytical utility of the aptasensor for a practical application. Detection of TET in milk is of considerable interest because milk is one of the most heavily regulated products in the food industry due to the risk of veterinary medicine residue contamination. The milk samples used in this study were all purchased from the RT-MART supermarket in the Zhangdian District of Zibo City, Shandong Province, China. The milk samples were diluted ten times with PBS. Further, a TET standard solution was spiked into the diluted milk, making for final concentrations of 5×10^{-9} M, 5×10^{-7} M and 5×10^{-5} M TET. Then experiments were carried out according to the aforementioned optimized conditions for TET detection with the developed aptasensor. The TET concentration recoveries were between 84%-92% (Table 1), which clearly indicate that the aptasensor was capable of detecting tetracycline in real milk samples.

Table 2. Testing results of antibiotic residue in milk samples

Sample	Origin of the milk samples	Original milk	Added (M)	Total found (M)	Recovery (%)
Deyi milk 1	The RT-MART supermarket	Not found	5×10^{-5}	4.5×10^{-5}	90
Deyi milk 2		--	5×10^{-7}	4.2×10^{-7}	84
Deyi milk 3		--	5×10^{-9}	4.3×10^{-9}	86
Yili milk 1		Not found	5×10^{-5}	4.6×10^{-5}	92
Yili milk 2		--	5×10^{-7}	4.3×10^{-7}	86
Yili milk 3		--	5×10^{-9}	4.2×10^{-9}	84

4. CONCLUSIONS

In this work, a novel, label-free aptasensor based on Fe_3O_4/IL for the detection of low levels of tetracycline was developed. The Fe_3O_4/IL gave the electrochemical aptasensor higher sensitivity due to the improved conductivity imparted by the ionic liquid and the thin film forming property of Fe_3O_4 magnetic nanoparticles. This combination significantly increased electron transfer between the electrode and the electrolyte, thus improving the detection limit of the sensor. The Fe_3O_4/IL system demonstrated good conductibility and biocompatibility and exhibited higher sensitivity and stability. Its electrochemical signal showed a linear relationship with TET concentration in the range of 1×10^{-2} M to 1×10^{-9} M. This strategy contributed to improved sensitivity and stability of the aptasensor, providing a novel, promising platform for TET detection.

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