Simple and Selective Colorimetric Detection of Oxytetracycline Based on Fe(III) Ion-3,3',5,5'-Tetramethylbenzidine

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Abstract

Oxytetracycline (OTC) is a common antibacterial agent used for the control of animal diseases. OTC abuse can seriously affect human health. Herein, based on the Fe(III)-3,3',5,5'-tetramethylbenzidine (Fe(III)-TMB) system, a facile and rapid colorimetric assay for oxytetracycline (OTC) was successfully developed. The addition of OTC could remarkably enhance the Fe(III)-oxidized TMB reaction and the absorbance increase of Fe(III)-TMB solution is proportional to the added OTC. The linear range of proposed sensor for OTC was from 20 nM to 1000 nM with the detection limit of 7.97 nM. The high sensitivity for OTC detection was successfully achieved under optimal conditions. For real sample analysis, recoveries of 89.93% to 100.02% was obtained. This is the first report for detecting OTC based on the nonenzymatic colorimetric reaction using the intrinsic oxidized activity of OTC/Fe³⁺ complex. The present simple, low-cost and visualized sensor has great potential for OTC detection in food.

Keywords

Colorimetric Detection, Oxytetracycline, Oxidation, Sensor

1. Introduction

Oxytetracycline (OTC) is a member of the most common broad-spectrum tetracycline (TCs) group of antibiotics. It has been widely used as veterinary drug and feed additive in livestock production for the treatment of infectious diseases and promote growth, due to its effective antimicrobial properties and low cost [1]. However, the inappropriate and illegal use of OTC has led to the OTC resi-

dues in some food and groundwater [2] [3] [4]. Long-term intake of food and drinking water containing OTC will cause serious threats to human health [5] [6]. Therefore, there have been extensive efforts to develop the sensitive and selective system for OTC detection in food products and water.

To date, various analytical methods have been proposed for the detection of OTC, such as chromatography methods [7] [8], fluorescence method [9] [10], and electrochemistry [11] [12], etc. Though most of these methods have high sensitivity, they suffer from the disadvantages of high costs, time-consuming, complicated sample pretreatment and sophisticated instrument manipulation, which limit their application for rapid, on-site and real-time determination. Therefore, the development of a simple, rapid and cheap method for detecting OTC has become increasingly attractive.

Compared with some other analytical methods, colorimetric analysis is simple and can be read out by the naked eye without the aid of sophisticated instruments, thus realizing the visual on-site analysis [13] [14] [15] [16]. There have been only a few reports about colorimetric OTC detection methods so far, which based on the formation of gold nanoparticles [13] or aggregation of gold nanoparticles [14] [17]. However, these sensing systems required synthesis and modifying gold nanoparticles for OTC detection. Thus, it is still a great challenge to establish more convenient and reliable label-free colorimetric strategies for the sensitive OTC detection. The chromogenic reaction between 3,3',5,5'-tetramethylbenzidine (TMB) and an oxidizing agent has been widely applied to the construction of label-free colorimetric sensing system [16] [18]. It has been reported that some ion possessing ability to oxidise TMB, such as Fe^{3+} , [19] Ag^+ , [16] ClO^- , [18] and produce a blue oxidation product. In this work, it was observed that the addition of OTC could remarkably enhance the Fe(III) -oxidized TMB reaction, which provided a colorimetric method for OTC detection. To the best of our knowledge, this is the first report for detecting OTC based on the nonenzymatic colorimetric reaction using the intrinsic oxidized activity of OTC/Fe^{3+} complex. Particularly, the present approach has been successfully applied to determine OTC in honey samples.

2. Experimental

2.1. Chemicals and Instrument

3,3',5,5'-tetramethylbenzidine (TMB), OTC and other antibiotics were obtained from Aladdin Industrial Inc. (Shanghai, China). FeCl_3 and other reagents were purchased from Beijing Chemical Reagent Factory (Beijing, China). All the reagents were used as received without further purification. All aqueous solutions were prepared with Milli-Q water ($>18.2 \text{ M}\Omega\text{-cm}$) from a Milli-Q Plus system (Millipore). UV-Vis detection was carried out on a PGENERAL T6 UV-Vis spectrophotometer (China).

2.2. OTC Detection

A stock solution of OTC (1 mM) was prepared in water and various concentra-

tions of OTC were obtained by serial dilution of the stock solution. For the detection of OTC, 250 μM of TMB, 250 μM of FeCl_3 , and OTC with different concentrations were added sequentially in 1 mL HAc-NaAc buffer solutions (0.1 M, pH 3.5). After that, the mixture was vortex mixed thoroughly and transferred for UV-vis scanning after incubating for 5 min at 20°C.

To elevate the selectivity of the proposed method, 250 μM of TMB, 250 μM of FeCl_3 , and 2 μM of kanamycin, streptomycin, chloramphenicol, doxitard, penicillin, tetracycline, vancomycin, neomycin, and OTC were added sequentially in 1 mL of 0.1 M NaAc buffer solutions (pH 3.5). UV-vis spectroscopy was recorded after incubating for 5 min at 20°C. Absorbance at 652 nm was used for quantitative analysis.

2.3. Pretreatment for the Analysis in Honey Samples

Honey samples were purchased from local supermarket and stored at room temperature before use. For removal of matrix effect, honey samples (1 g) were mixed with 5 mL of Mc Ilvaine buffer containing 20 mM EDTA (pH 5.0) and 0.5% (v/v) trifluoroacetic acid for 5 min using a vortex mixer, then homogenized by ultrasonic for 5 min, and centrifugated at 4°C for 20 min at 4000 rpm. Then 4 M NaOH was added drop wisely to the supernatant to adjust the pH to 7.6. For the final test, the above sample was diluted 2000 times.

3. Results and Discussion

3.1. OTC Enhanced the Fe(III)-TMB Reaction

To demonstrate the feasibility of our approach, the TMB oxidation was first studied in different systems (**Figure 1(a)**). As illustrated in **Figure 1(a)**, the Fe^{3+} can oxidize TMB to produce a blue color with a maximum absorbance at 652 nm (**Figure 1(a)(2)**). After the addition of OTC to Fe^{3+} -TMB system, a dramatic

Figure 1. (a) UV-vis spectra of obtained solutions under different conditions (followed by bottom-up): TMB, TMB + OTC, TMB + Fe^{3+} , and TMB + OTC + Fe^{3+} in pH 3.5 NaAc-HAc buffer at 20°C. Inset: corresponding photographs. (b) Effect of Fe salts on absorbance change in the presence or absence of 20 μM OTC. The concentrations of Fe salts were 250 μM .

increase of absorbance is observed (**Figure 1(a)(4)**). However, OTC has no appreciable effect on the oxidation of TMB. Therefore, we speculate that a OTC-Fe³⁺ complex could be formed after the addition of OTC to Fe³⁺, which would enhance the Fe³⁺-TMB reaction. In order to optimize the reaction system, we have chosen different Fe salts to oxidize TMB (**Figure 1(b)**). It is found that FeSO₄ cannot oxidize TMB, even after OTC was added into the Fe²⁺-TMB solution (**Figure 2**). Adding OTC to FeCl₃-TMB or Fe(NO₃)₃-TMB mixtures lead to the close absorbance increase, but the ferric acetylacetonate (Fe(acac)₃)-TMB system shows a remarkable decrease in peak absorbance after adding OTC (**Figure 1(b)**). Thus, FeCl₃ was used as Fe³⁺ source in the following studies.

3.2. Optimization of Detection Conditions

To improve the sensitivity of the established colorimetric system, experimental conditions for OTC detection were carefully optimized at first. The reduction of absorbance ($\Delta A = A - A_0$) was used as a criterion to optimize the detection conditions, where A and A_0 represent the absorbance at 652 nm in the presence and absence of OTC, respectively.

3.2.1. Effect of pH

pH is an crucial factor for almost every sensing system. The pH effect for OTC detection was firstly studied as shown in **Figure 3(a)**. It is shown that ΔA increase with increasing pH value in the range from 2.0 to 3.5. Further increase of pH value results in the decrease of ΔA . Therefore, pH 3.5 was adopted in the following experiments.

3.2.2. Effect of Reaction Temperature and Reaction Time

Figure 3(b) and **Figure 3(c)** show the effect of reaction temperature and reaction time on the Fe³⁺-TMB sensing system. Different incubation times ranging from 5 min to 30 min and temperature ranging from 20 °C to 50 °C were investigated to

Figure 2. UV-vis spectra of 3,3',5,5'-tetramethylbenzidine solution with different Fe salts in the presence or absence of 20 μM oxytetracycline (OTC). The concentrations of Fe salts were 250 μM.

Figure 3. Effect of pH (a), reaction temperature (b), reaction time (c), Fe^{3+} concentration (d) and TMB concentration (e) on the ΔA . (f) is the effect of metal ions on absorbance in the presence or absence of OTC.

study their effects on the ΔA . The results indicated that the highest ΔA was obtained at 5 min when the reaction temperature was fixed at 20°C.

3.2.3. Effect of Fe^{3+} and TMB Concentrations

The effects of TMB and Fe^{3+} concentrations on the ΔA were displayed in **Figure 3(d)** and **Figure 3(e)**, respectively. The experiments show that the maximum of ΔA was obtained when the TMB concentration was fixed at 250 μM and the Fe^{3+} concentration was 250 μM .

3.2.4. Effect of Different Metal Ions

An experiment was also carried out to examine the effect of different metal ions on OTC detection. According to the comparison of absorbance with or without 0.5 μM OTC under different 50 μM metal ions (**Figure 3(f)**), Fe^{3+} shows the best catalytic activity towards the TMB solution to produce an absorbance increase and the other metal ions have no obvious catalytic activities. This result indicates that Fe^{3+} is the favored metal ion for constructing OTC sensing system.

3.3. Colorimetric Detection for OTC

As shown in **Figure 4(a)**, the ΔA gradually increased with increasing OTC concentration and a good linear relationship was found between ΔA and OTC concentration under the optimum conditions. The linear correlation of $\Delta A = 0.023 + 0.469 \times C[\text{OTC}]$ ($R^2 = 0.985$) was obtained over the tested concentration range of OTC from 20 - 1000 nM. The limit of detection (LOD) was calculated to be 7.97 nM (3.96 $\mu\text{g/L}$) at a signal-to-noise ratio of 3. The LOD is better than those of some reported methods, [20] [21] [22] such as colorimetric aptasensor (25 nM) [17], electrochemiluminescence sensor (0.1 μM) [23], colorimetric detection (0.0838 $\mu\text{g/mL}$) [24], and aptasensor (12.3 $\mu\text{g/L}$) [25].

3.4. Selectivity of Sensor

To further examine the selectivity of this proposed sensor for OTC, the responses of system to eight other antibiotics including kanamycin, streptomycin, chloramphenicol, doxitarid (DOX), penicillin, tetracycline (TC), oxytetracycline, vancomycin, and neomycin were studied. With 20 μM analytes, OTC causes the maximum ΔA in the sensing system as shown in **Figure 5**. However, the addition of TC and DOX also leads to slightly increase of absorbance comparing with other antibiotics. We assume that TC, DOX and OTC are antibiotics of the tetracycline group and have similar structures. The results demonstrate that our sensor is highly selective toward OTC over other classes of antibiotics.

3.5. OTC Detection in Honey Sample

To investigate whether the colorimetric method can be used in real samples analysis, the OTC detection in honey sample was studied (**Table 1**). It can be seen that the recoveries vary from 89.93% to 100.02%, indicating the proposed colorimetric assay is highly reproducible and accurate for rapid detection of OTC.

4. Conclusion

In summary, based on the Fe^{3+} -TMB system, a facile and rapid colorimetric

Figure 4. Dependence of the absorbance change at 652 nm, temperature 20 °C and pH 3.5 on the concentration of OTC (a) and the corresponding linear calibration plots (b).

Table 1. Colorimetric determination of OTC in honey samples ($n = 3$) by Fe(III)-TMB system.

Honey sample	Spiked concentration (nM)	Measured concentration (nM, mean)	Recovery (% , mean)	RSD (% , $n = 3$)
Sample 1	0	8.98		6.211
	500	478.69	93.94	2.116
	1000	989.34	98.04	8.769
Sample 2	0	63.67		1.166
	500	513.32	89.93	3.473
	1000	1063.93	100.02	3.907

Figure 5. The ΔA response of the sensing system toward OTC and other antibiotics.

sensing system for OTC detection was successfully fabricated. The linear dynamic range for OTC was found from 20 nM to 1000 nM with the detection limit of 7.97 nM. The sensor for OTC detection showed high sensitivity under optimal conditions. When used the system to honey sample, recoveries varying from 89.93% to 100.02% were obtained. This assay is lowcost and convenient. Furthermore, the proposed method is promising for the analysis of OTC in foods.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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