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Sensitive detection of chloramphenicol based on Ag-DNAzyme-mediated signal amplification modulated by DNA/metal ion interaction

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ABSTRACT

We here report a novel method for antibiotic detection by making use of DNA/metal ion interaction coupled with Ag-DNAzyme cleavage-mediated signal amplification. Taking the analysis of chloramphenicol (CAP) as an example, upon the specific recognition between the antibiotic CAP and its aptamer, the secondary structure of the DNA aptamer shaped by C-Ag^+-C base mismatches will be altered, liberating the pre-captured Ag^+. Subsequently, the free Ag^+ provided as a cofactor can activate the Ag-DNAzyme, which behaves recycled cleavage of substrate DNA on the electrode surface for signal amplification. The more CAP is present, the more Ag^+ is released, thus more Ag-DNAzyme can be activated to achieve a higher electrochemical signal. Therefore, the target-responsive variation of electrochemical signal enables the sensitive detection of CAP. The proposed method is cost-effective only with plain metal ion as modulator. It has also been challenged with real food samples, indicating the potential to be a promising tool for food safety detection.

1. Introduction

Antibiotics have been broadly applied in both human and animal medicine with good pharmacokinetic properties (Lam and Crawford, 2018; Wright, 2014). However, over amount of antibiotics obtained from food or medicine may bring about many serious adverse effects on human beings, such as decline of immunity and resistance to microorganisms (Zeissig and Blumberg, 2014). Chloramphenicol (CAP), a broad-spectrum antibiotic, has been extensively employed to the treatment of food-producing animals as a low-cost and highly effective drug (Cui et al., 2016; Rose et al., 2005). As a result, a trace amount of CAP could gather in meat or milk, and may be accumulated in the body of human by the food chain, resulting in potential risk to human health (Crump et al., 2015; Kohanski et al., 2010). Thus, development of method to efficiently detect antibiotics residues such as CAP has received widespread attention. Existing methods for the detection of CAP (Chang et al., 2017; Kaufmann et al., 2015; Pan et al., 2015; Wu et al., 2015), such as chromatography, immunology, fluorescence and microgravimetry, may need complicated sample preparation, expensive instrumentation and laborious procedures. Therefore, there has been increasing interest in the development of novel method with high sensitivity, accuracy and simplicity to detect CAP residues for food safety and clinical diagnosis.

The interaction of metal ions with nucleic acids has been explored with a great deal of interest (Funai et al., 2014; Ono et al., 2008). In particular, metal ion-mediated base pairs, formed by the specific binding of metal ions to natural or artificial bases in a way that is different from natural complementary nucleobases, impart nucleic acids with unique chemical and physical properties, such as the increasement of duplex and triplex stability and the formation of DNA-assembly arrays (Chen et al., 2016; Guo et al., 2014). Representative examples of metal ion-mediated base pairs are the coordination of Hg^{2+} and Ag^+ to thymine-thymine (T-T) and cytosine-cytosine (C-C) respectively, leading to the formation of the T-Hg^{2+}-T and C-Ag^+-C base mismatches (Miyake et al., 2006; Zheng et al., 2014). Recently, many researches have concentrated on another novel typical example, a kind of metalloenzyme called DNAzyme, which are composed of DNA molecule and can catalyze cleavage of its nucleic acid substrates by selectively incorporating of metal ions as cofactors (Huang et al., 2015; Torabi et al., 2015). Merits including excellent catalytic performance, high metal ion selectivity, flexible design and modification, as well as economic synthesis make DNAzyme an attractive candidate for developing biosensors (Gong et al., 2015; Lilienthal et al., 2015; Seok et al., 2015). However, many of these kinds of biosensors usually require

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complicated designs for the operational system or complex control for enzyme activity switch, and the involvement of multiple chimeric DNAs to play important roles, leading to high construction costs and limited detection abilities (such as sensitivity and specificity). On the other hand, even though metal ion-mediated base pairs have been widely implemented for the construction of nanodevices and the development of sensing platforms for the detection of divalent metal ions (Zhang et al., 2016), proteins (Yang et al., 2016), and nucleic acids (Peng et al., 2017), very few attempts have been made towards antibiotics assays by using metal ion-mediated base pairs.

In this work, we have developed a sensitive method for CAP antibiotic detection by making use of DNA/metal ion interaction. The metal ion, Ag^+, utilized here can trigger the formation of new secondary structure of the DNA aptamer through C-Ag^+-C base mismatches. While the specific interaction of target CAP with its aptamer will alter the secondary structure of the DNA aptamer and release the captured Ag^+. Consequently, the free Ag^+ can activate the newly formed Ag^+-dependent DNAzyme (Ag-DNAzyme) (Saran and Liu, 2016) that can exhibit recycled cleavage of its nucleic acid substrates and thus amplify the signal of electrochemical response. The more target CAP exists, the more Ag^+ is freed and the higher electrochemical signal is obtained. Therefore, the target CAP detection can be realized through monitoring the change of electrochemical signal. This assay method depends on the target-responsive signal generation, transduction and amplification, which is not only specific and sensitive but also very cost-effective in design only with plain metal ion as modulator. The assay of CAP residues in real foodstuff samples has also been conducted although they may contain complex matrix, obvious background noise and low concentrations of CAP, indicating potential practical application.

2. Experimental section

2.1. Materials and reagents

CAP, 3-(N-morpholino)-propanesulfonic acid (MOPS), mercaptohexanol (MCH), Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), $NaNO_3$ and $AgNO_3$ were purchased from Sigma-Aldrich. Oligonucleotides used here were synthesized and purified by Sangon Biotechnology Co. Ltd. (Shanghai, China), and the sequences were as follows: Aptamer (5′-ACTTCAGTGAGTTGTCCCACGGTCGGCGAGTCG GTGGTAG-3′), Ag-DNAzyme (5′-GCTTCGCCATCTTTAGGTGATTTCCA CGATTATGCGGAAACAGGGCAGCGTATAGTGAG-3′), Substrate DNA (5′-SH-CTCACT ATrAGGAAGATGGCGAAGCAAAAAAAAAAGGGGGGT TTTTTTTTT-3′). The other chemicals in this work were of analytical grade and directly used without additional purification. All solutions were prepared with ultrapure water (> 18.0 MΩ) from a Millipore system. The buffer solutions used in this work are listed as follows. DNA immobilization buffer: 50 mM MOPS, 1 mM EDTA, 10 mM TCEP, and 100 mM $NaNO_3$ (pH 7.0). Reaction buffer: 50 mM MOPS containing 200 mM $NaNO_3$ (pH 7.0). Milk sample purchased from local supermarket was pretreated for analysis. The Milk sample was first centrifuged at 10000 rpm for 10 min (10 °C) to eliminate the upper fat, then 10% (v/v) perchloric acid aqueous solution was added for protein precipitation with several repetitions, and next the supernatant was collected and adjusted to appropriate pH. Finally, the supernatant was vortexed and centrifuged at 7000 rpm for 2 min. The prepared supernatant was ready for assay.

2.2. Gold electrode treatment and modification

The steps of gold electrode treatment were almost the same as our previous work (Huang et al., 2016). Briefly, the gold electrodes (3.0 mm) were first immersed in freshly prepared piranha solution (H_2SO_4: 30% H_2O_2 = 3:1) for 5 min, followed by rinsed with double-distilled water. Then the electrodes were polished with 1 μm and 0.3 μm alumina slurry on a microcloth in sequence. Finally, the electrodes were sonicated in both ethanol and double-distilled water for 5 min to remove residual alumina powder, and electrochemically cleaned with 0.5 M H_2SO_4.

After being dried with nitrogen, the prepared electrodes were incubated with 3 μM substrate DNA (pretreatment with 10 mM TCEP for 1 h to cleave disulfide bonds) at room temperature for 2 h, followed by being immersed in an aqueous solution of 1 mM MCH for 2 h at room temperature to make the oligonucleotides in good order. The modified electrodes were then further rinsed with double-distilled water and dried again with nitrogen.

2.3. Detection of CAP

For the detection of CAP, Ag^+ (10 μM) was mixed and reacted with the anti-CAP aptamer (1 μM) and incubated at room temperature for 90 min. Then, CAP solutions of different concentration were added into the mixture and incubated for 45 min at 37 °C to release previously captured Ag^+. After that, the substrate modified electrode was immersed into the above mixture with 2.5 μM Ag-DNAzyme, followed by incubation at 25 °C for 30 min to allow DNAzyme to cleave the substrate. Finally, the electrode was gently rinsed with double-distilled water and the response of electrochemical signal was recorded.

2.4. Electrophoresis mobility shift assay

Gel electrophoresis analysis of 5% agarose gel was employed to demonstrate the assay reaction. Electrophoresis was carried out with MOPS buffer (20 mM MOPS, 5 mM NaAc, 1 mM EDTA, pH 7.0) as running buffer, and with a 100 V constant voltage at room temperature for 50 min. After separation, visualization was conducted using the fluorescence gel imaging system after stained with GelRed.

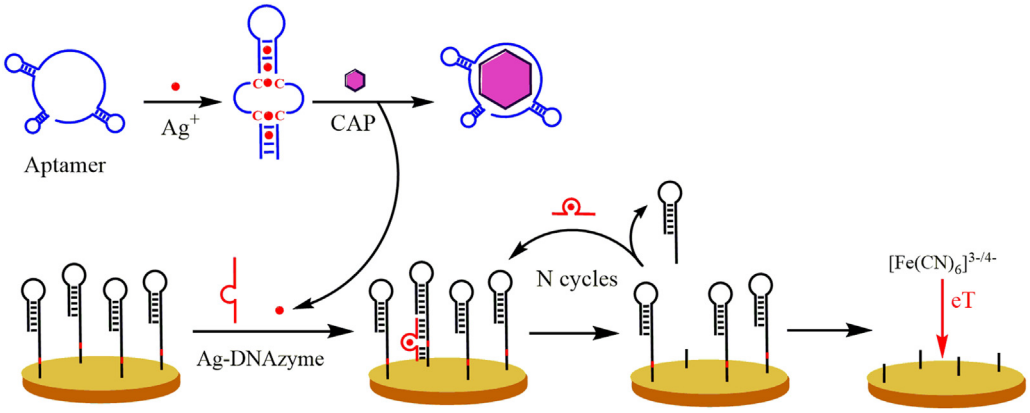
2.5. Electrochemical measurements

All the electrochemical measurements were conducted on a CHI660D electrochemical workstation (CH Instruments, USA) at room temperature. Square wave voltammograms (SWVs) and electrochemical impedance spectra (EIS) were recorded with a conventional three-electrode system, which included a modified gold electrode as the working electrode, a platinum wire as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode, respectively. SWVs and EIS were recorded in 5 mM $[Fe(CN)_6]^{3-/4-}$ with 1 M KNO_3. Experimental parameters were as follows. SWV, scan range, − 0.1 ~ 0.6 V, step potential, 5 mV, frequency, 15 Hz, amplitude, 25 mV. EIS, bias potential, 0.224 V, frequency range, 0.1 Hz ~ 10 kHz, amplitude, 5 mV. The data were obtained by independent experiment performed in triplicate.

3. Results and discussion

3.1. Detection principle

The principle of this sensing strategy for CAP detection has been illustrated in Scheme 1. Ag^+ selectively binds to C-C mismatches and induces the formation of new secondary structure of the anti-CAP DNA aptamer. In the presence of target CAP, the specific interaction of CAP with its aptamer will distort the secondary structure of the DNA aptamer and result in the release of captured Ag^+. The free Ag^+, as a cofactor, can activate the Ag-DNAzyme. On the other hand, a hairpin DNA containing the substrate sequence of the Ag-DNAzyme is pre-immobilized onto a gold electrode via gold-sulfur chemistry. With the existence of Ag^+, the DNAzyme hybridizes with the surface-tethered substrate sequence and effectively cleaves it at 25 °C. Due to the low melting temperature (T_m < 25 °C) between DNAzyme with the cleaved-product sequence on electrode surface, the hairpin DNA sequence is removed along with DNAzyme from the electrode surface. After



Scheme 1. The schematic illustration of the proposed method for CAP detection. Not drawn to scale.

cleaving one surface-tethered DNA, the DNAzyme sequence can hybridizes with another DNA substrate, starting a new DNA cleavage cycle and resulting in considerable signal amplification eventually. The more target CAP exists, the more surface-tethered DNA is eliminated, leading to larger electrochemical response of $[Fe(CN)_6]^{3-/4-}$. Therefore, the final signal response is positively relevant to the concentration of target CAP, and the sensitive detection of target antibiotic can be achieved.

3.2. Validation of detection principle

As an efficient and facile tool for investigating the interface properties of modified electrode, electrochemical impedance spectroscopy is employed to validate the stepwise treatment and modification of the electrode. In electrochemical impedance spectra (EIS), the increase of semicircle diameter may be corresponding to the increase of interfacial charge transfer resistance. As shown in Fig. 1A, the impedance spectrum of bare gold electrode presents as a straight line (curve a, 3 Ω). With the modification of substrate DNA on the electrode, a large semicircle diameter appears (curve b, 2225 Ω), suggesting the increasement of interfacial electron transfer resistance due to the DNA immobilization. Further modification of MCH to form self-assembled mono-layer on the electrode leads to a slightly larger semicircle diameter (curve c, 2300 Ω). After incubation with the reaction mixture containing target CAP, the diameter of the semicircle obviously decreases (curve d, 500 Ω), since the activated Ag-DNAzyme can cleave the substrate DNA and consequently reduce immobilized DNA strands on the electrode surface. In addition, electrochemical impedance spectroscopy is implemented to examine the target-responsive reaction. As shown in Fig. 1B, a much smaller interfacial electron transfer resistance is obtained in the presence of target CAP (curve a), compared with the negative control (curve b). The target-responsive reaction has been further verified by agarose gel electrophoresis analysis (Fig. 1B, inset). The negative control without CAP (lane 1) exhibits two bands corresponding respectively to the aptamer of CAP (40 nt) and the complex of Ag-DNAzyme and substrate DNA (109 nt). In the presence of CAP (lane 2), besides an obvious band corresponding to the aptamer of CAP (40 nt) and the cleaved-DNA product (41 nt), a new characteristic band of the cleaved-DNA product (9 nt) can also be observed. These results show that the reaction system is triggered and initiated by target CAP. Moreover, to further validate the detection principle, control experiment without Ag^+ is carried out. As present in Fig. S1, with Ag^+ as a cofactor, Ag-DNAzyme can be activated and cleaved the substrate DNA on the electrode surface, leading to an obvious decrease in the charge transfer resistance (curve a) compared with the negative control without Ag^+ (curve b). It proves that the decrease in the charge transfer resistance is caused by the cleavage of the substrate DNA by Ag-DNAzyme.

3.3. Optimization of experimental conditions

Experimental conditions play important roles for achieving the best analytical performance, so several experimental conditions are then optimized. Firstly, a higher concentration of Ag^+ leads to a stronger electrochemical response, however, a relatively high background signal is also generated with the increase of Ag^+ concentration, so the concentration of Ag^+ is optimized. As presented in Fig. S2, the value of I/I_0 shows a peak-shape dependency with the Ag^+ concentration. According to the highest ratio of I/I_0, the optimal concentration of Ag^+ is selected as 10 μM. In addition, the temporal response of the detection system with the incubation time of target CAP is also examined. A time-dependent change of electrochemical signal is shown in Fig. S3. The electrochemical response increases continuously and almost reaches a plateau at the incubation time of 45 min, so 45 min is sufficient. To investigate the effect of temperature, the electrochemical response with different incubation temperature of target CAP is recorded. As present in Fig. S4, the optimal incubation temperature for CAP with the

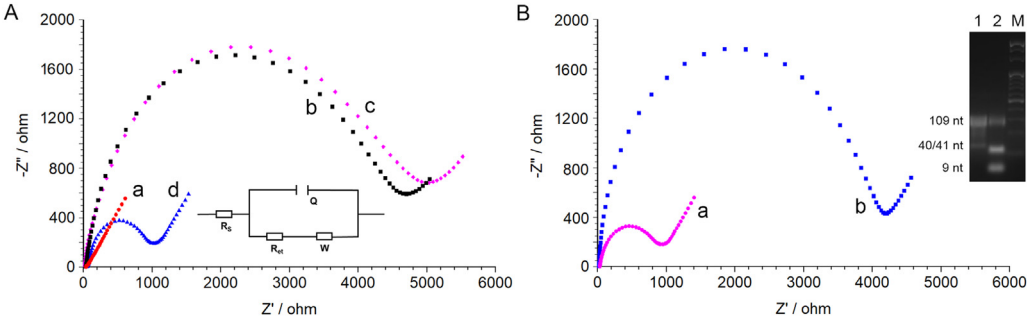


Fig. 1. Feasibility study of the proposed method. (A) EIS recorded to validate the stepwise treatment of the electrode, (a) the bare gold electrode, (b) the electrode after modification with substrate DNA, (c) after modification with MCH, (d) after incubation with the mixture sample containing 40 ng mL^{-1} CAP. Inset is the equivalent circuit, Rs, Ret, W and Q represent the solution resistance, the charge-transfer resistance, the Warburg impedance and the constant phase element, respectively. (B) EIS corresponding to (a) experiment with CAP, (b) experiment with no CAP. Inset is agarose gel electrophoresis verification of the method, (1) experiment with no CAP, (2) experiment with CAP.

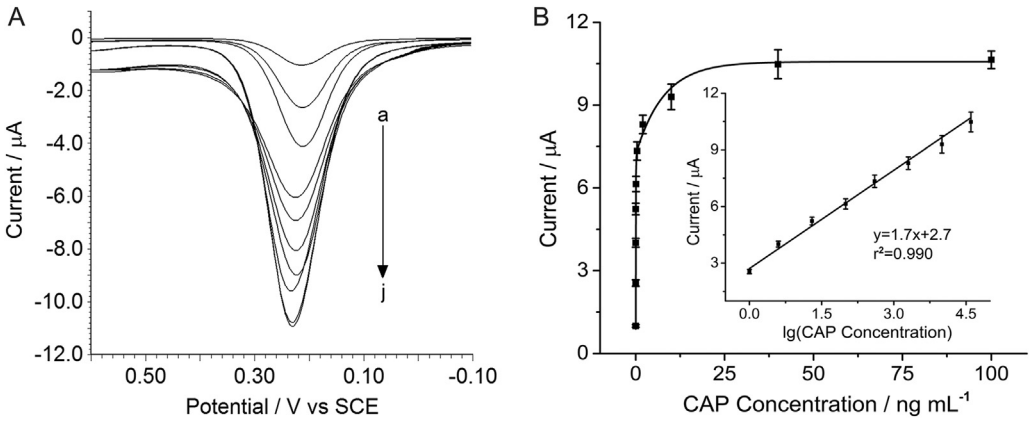


Fig. 2. The analytical performance of the proposed method for CAP detection. (A) Square wave voltammetry responses of the method for various concentrations of target CAP, a ~ j (pg mL^{-1}): 0.4, 1, 4, 20, 100, 400, 2000, 10000, 40000, 100000. (B) Calibration curve of the method for target CAP. Inset shows the linear range. The error bars represent the standard deviation of three repeated experiments.

aptamer is 37 °C according to the highest electrochemical signal. Moreover, both the concentration and reaction time of Ag-DNAzyme play vital roles in signal amplification for the detection of CAP. As shown in Fig. S5, the electrochemical signal increases gradually as the reaction time prolongs at various concentrations of DNAzyme, and the maximum current is obtained at 30 min with the concentration of 2.5 μM. Therefore, 30 min and 2.5 μM are chosen as the optimal time and optimal concentration of DNAzyme respectively for the assay experiment.

3.4. Performance of the detection method

Under the optimized conditions, electrochemical responses of the assay method toward CAP standards have been investigated. Fig. 2 depicts the SWVs recorded by analyzing CAP at different concentrations. The peak currents increase with the increase of CAP concentration, and the linear relationship between the peak current and the logarithmic value of CAP concentration is found from 1 pg mL^{-1} to 40 ng mL^{-1}, while a detection limit (LOD) is calculated to be 0.4 pg mL^{-1} based on the criterion of signal-to-noise ratio = 3. These results could fully indicate that the developed method exhibits satisfactory sensitivity with a very low LOD and a large linear range for CAP. Each result represents the average of three repetitive measurements. To be noted, the analytical performances of the proposed method is comparable with or even more superior than some previously reported methods (Table S1). To demonstrate the specificity of the proposed assay method for CAP, several control antibiotics, including thiamphenicol (TAP), florfenicol (FF) and erythromycin (ERYC), are employed to challenge this assay method. As seen in Fig. 3, under the same experimental conditions, appreciably higher signal response can be observed with the target CAP compared with the other antibiotics, and the existence of the potential interference antibiotics does not influence the signal response of the assay method for the target CAP. These results may clearly reveal the high specificity of the proposed method.

CAP as a broad-spectrum antibiotic has been widely applied to the treatment of food-producing animals, which may result in its accumulation in foodstuff and the ultimate accumulation in the body of human, leading to potential risk for human health. Therefore, the monitoring of CAP from complex food samples is highly important for food safety detection. CAP in milk samples have been measured by using the proposed method to examine its potential application in real samples analysis. A series of CAP concentration including 5, 50, 500, and 5000 pg mL^{-1} are prepared by adding CAP standards into milk samples and assayed via the proposed method. As shown in Table 1, the recoveries are from 92% to 110% and the corresponding relative errors are also admissible, suggesting fine accuracy. These results adequately demonstrate the potential application of the proposed method for CAP assay in complicated real samples.

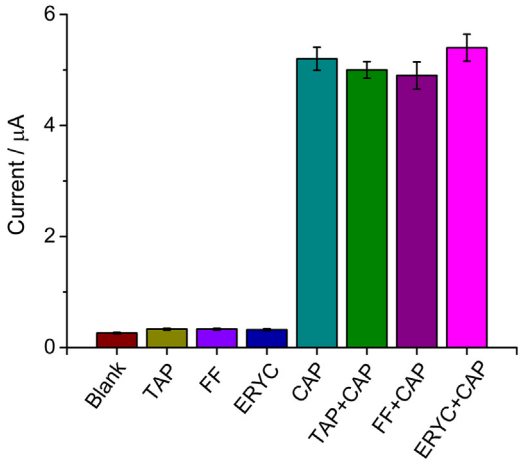


Fig. 3. Specificity study of the proposed method for target CAP. Electrochemical response of the method to different antibiotics and to target CAP in the presence of other control antibiotics. Blank control: 50 mM MOPS (pH 7.0). All the antibiotic concentrations are at 20 pg mL^{-1}. All the experimental conditions are the same as the optimized conditions. The error bars represent the standard derivation of independent experiments performed in triplicate.

Table 1
The recoveries of the proposed method for CAP assay in milk samples.

Added (pg mL^{-1})	Detected (pg mL^{-1})	Recovery (%)	Relative error (%)
5	5.5	110	3.8
50	46	92	4.4
500	510	102	5.6
5000	4800	96	4.5

Each concentration was measured for three independent experiments.

4. Conclusions

In conclusion, based on DNA/metal ion interaction coupled with Ag-DNAzyme cleavage-mediated signal amplification, a novel sensing strategy is proposed for antibiotic CAP assay. This assay method is not only specific and sensitive but also has the feature of cost-effectiveness. This work has also been validated to be a feasible electrochemical sensing platform for detection of CAP in real food sample. Moreover, this work can be extended for the detection of other antibiotics by simply replacing the corresponding aptamers. Certainly, since the aptamers of some antibiotics have not been known, there are also some limitations of the sensing strategy proposed in this work. Nevertheless, with the screening of the aptamers for other antibiotics, more biosensors will be developed for the analysis of other antibiotics.

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Declaration of interests

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2018.12.016.

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