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Electrochemical Aptasensor based on the Hybridization Chain Reaction for Kanamycin Detection

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In this work, a novel electrochemical aptamer sensor was fabricated for the sensitive and label-free detection of kanamycin in milk. The kanamycin aptamer was fixed on the magnetic nanoparticle for capturing kanamycin or complementary probe. The complementary probe and a pair of hairpin probes were used as initiators for the hybridization chain reaction. Methylene blue was used as electroactive indicator for electrochemical detection. Under optimal experimental conditions, the aptamer sensor has a good linear relationship with kanamycin concentration in the range of 1.0×10^{-12} to 1.0×10^{-7} mol/L. The detection limit of the aptamer sensor is 1.0×10^{-12} mol/L (S/N = 3). The proposed method can be successfully applied for the detection of kanamycin in milk.

Keywords: kanamycin; aptamer; hybridization chain reaction

1. INTRODUCTION

As a member of aminoglycoside compound, kanamycin, which exhibits strong ability against a series of diseases, has been widely used in animal husbandry, aquaculture and other fields [1]. However, the abuse of kanamycin may result in its accumulation in animal-derived food (e.g., meat and milk). If such food is consumed for a long time, consumers may suffer from side effects such as ototoxicity and nephrotoxicity [2, 3]. At present, the content of kanamycin in food has been clearly ruled in many countries. The latest policy in China stipulates that the residue of kanamycin should not exceed 150 and 100 μg/kg in animal dairy products and animal muscles respectively [4].

The common methods for the detection of kanamycin include high-performance liquid chromatography (HPLC) [5, 6], microbial [7], enzyme-linked immunosorbent assay (ELISA) [8] and HPLC-mass spectrometry (HPLC-MS) [9, 10]. These techniques have many advantages, such as low

detection limit and high precision. However, some drawbacks, including time-consuming, low selectivity, and the requirement of expensive apparatus, remain. Therefore, efficient methods for kanamycin determination with high sensitivity and selectivity should be explored [11].

Aptamers are usually less than 100 oligonucleotide fragments that are obtained by an in vitro selection progress called systematic evolution of ligands by exponential enrichment, and can be specifically combined with enzymes, proteins, metal ions and organic matter [12]. Given the advantages of easy synthesis, strong specificity, low cost and good affinity, aptamers are applied in electrochemical detection [13, 14], fluorescence assays [15], cantilever assay [16], and spectrophotometry [17]. Many researchers attempted to combine aptamer with signal amplification methods for trace analysis for the improvement of detection performance. The most used signal amplification methods include polymerase chain reaction [18], rolling circle amplification [19], loop-mediated isothermal amplification [20] and hybrid chain reaction (HCR). These methods have high specificity and high flux [21]. Notably, HCR possesses prominence of isothermal, enzyme-free reaction and high amplification efficiency. Currently, HCR has been applied in biological detection and trace analysis. Tang et al [22] designed an enzyme-free fluorescence sensor based on graphene and HCR, with detection limit for Staphylococcus aureus in milk of 4.00×10^2 CFU/mL. Bai et al [23] developed an aptamer sensor combined HCR with G-quadruplex DNAzyme for carcinoembryonic antigen detection, and its detection limit is 0.20 nmol/L.

Herein, an electrochemical aptamer sensor coupled with HCR amplification is established for the detection of kanamycin. In this sensor, the kanamycin aptamer, which can specifically recognize kanamycin, is immobilized on magnetic nanoparticles (MNPs) as capture probe. The complementary probe (CP) that has sequences complementary to the kanamycin aptamer is designed for HCR amplification and two hairpin DNA probes (i.e., H1 and H2). The aptasenor is expected to be successfully applied in the determination of kanamycin residue in complex food specimens.

2. MATERIALS AND METHODS

2.1 Materials and reagents

MNPs modified with streptavidin was purchased from Enrichin Inc (Shanghai, China), Methylene blue (MB) was supplied by Kelvin Biochemistry Co., Ltd. (Shanghai, China). Kanamycin was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Tetracycline, tobramycin and ampicillin were obtained from Diamond (Shanghai, China). All solutions in the experiment were prepared using ultra-pure water (>18.2 MΩ·cm).

According to the reference [24], all DNA sequences were synthesized by Sangon Biotech (Shanghai, China). Sequences are detailed in Table 1.

Table 1. DNA sequences

Name	sequences(5′→3′)
Kanamycin Aptamer	biotin-AAAAAATGGGGGTTGAGGCTAAGCCGA

CP	TAACAAGAAAGCCAAACCGAGATGCACTGTCGGCTTAGCCT
H1	CATCTCGGTTTGGCTTTCTTGTTACCCAGGTAACAAGAAAGCC AAACCC
H2	TAACAAGAAAGCCAAACCGAGATGGGTTTGGCTTTCTTGTTA CCTGGG

*The red parts represent binding sites.

2.2 Main instrument

Electrochemical measurements were performed on the CHI660e electrochemical workstation (Shanghai, China) with a conventional three-electrode system. This workstation consisted of magnetic glassy carbon electrode as working electrode, saturated calomel electrode as reference electrode, and platinum electrode as counter electrode. All electrodes were obtained from GOOSS UNION (Wuhan, China).

2.3 Synthesis of kanamycin aptamer-coated MNPs

In brief, 20 μL of 10 mg/mL streptavidin-modified MNPs were transferred into a 2 mL centrifuge tube. After thoroughly washing thrice with 1 mL Tris-HCl buffer (5 mmol/L, pH = 7.4), MNPs were mixed with 100 μL of 1 μmol/L kanamycin aptamer. The mixture was stirred and reacted at room temperature (25 °C ± 1 °C) for 30 min in the dark. During this process, streptavidin linked MNPs with the biotinylated aptamer. Subsequently, the resulting MNPs were easily separated using a magnet, and the supernatant containing the free kanamycin aptamer was removed. After washing thrice with Tris-HCl buffer, kanamycin aptamer-coated MNPs were resuspended in 1 mL of 5 mmol/L Tris-HCl buffer (pH = 7.4) and stored in the dark at 4 °C for further experiment.

2.4 Detection procedure for kanamycin

About 100 μL of 2 μmol/L CP and kanamycin were added and mixed with kanamycin aptamer-coated MNPs. The reaction was left to proceed for 30 min at room temperature in the dark under stirring. Then, resulting MNPs were thoroughly rinsed thrice with Tris–HCl buffer to remove excess CP. Next, 50 μL of 3 μmol/L H1 and H2 were added for HCR amplification. The HCR was conducted for 30 min at room temperature followed by washing thrice with Tris–HCl buffer to remove the surplus hairpin.

Prior to electrochemical detection, the magnetic glass carbon electrode was polished with 1.0, 0.3, and 0.05 μm aluminum slurry until its surface was smooth without damage. Subsequently, the magnetic glassy carbon electrode was cleaned with 50% (*v/v)* ethanol solution, 3:1 (*v/v*) nitric acid solution, ultrapure water and dried with nitrogen. The electrode was then activated from −0.2 V to 1.4 V in 0.5 mol/L H_2SO_4 at a scan rate of 0.1 V/s for 20 cycles until stable cyclic voltammetry curves were obtained. Afterwards, the electrode was cleaned with ultra-pure water and dried using high-purity nitrogen. After pretreatment, the magnetic glass carbon electrode was used to absorb the resulting MNPs on the detection surface by its own magnetic force. The differential pulse voltammetry (DPV) was

performed in 0.01 mol/L PBS buffer solution (pH = 7.0) at room temperature with the following conditions: potential range of 0.1 V to − 0.5 V with pulse amplitude of 0.05 V, pulse width of 0.05 s and quiet time of 2 s.

The content of kanamycin was quantified by measuring the net current intensity (ΔI, the difference of the current intensities with and without the addition of kanamycin).

2.5 Detection of kanamycin in food samples

Six concentrations of kanamycin in spiked milk were analyzed using the proposed method. Specimens were pretreated in accordance with the reference [25]. First, 20% trichloroacetic acid solution was used to adjust pH to 4.5. After incubation for 20 min under 45 °C, samples were centrifuged at 10000 rpm for 20 min. The supernatant was collected and filtered with 0.22 μm ultrafiltration membrane for subsequent detection.

3. RESULTS AND DISCUSSION

3.1 Principle of this electrochemical aptasensor

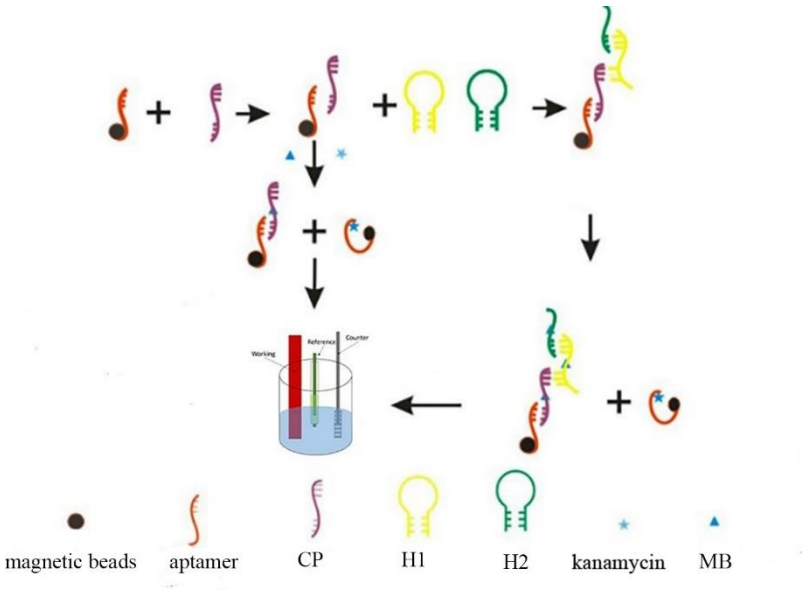


Figure 1. Principle of the proposed method

The principle of our HCR-based electrochemical detection method is depicted in Figure1. The kanamycin aptamer was immobilized on MNPs by using the streptavidin-biotin reaction. In the absence of kanamycin, free CP can directly hybridize with kanamycin aptamer anchored on the surface of MNPs to form double-stranded DNA (dsDNA) oligonucleotides. With the further addition of hairpin nucleic acid monomers (i.e., H1 and H2), HCR can be triggered by the fixed initiator (i.e., CP), producing a

long-chain dsDNA. Therefore, MB can intercalate into the long nicked double-helix, and act as electroactive indicator, thereby acquiring a strong electric signal. Instead, if kanamycin exists in a sample, the kanamycin aptamer tends to recognize the analyte specifically to form the target-aptamer complex. Hence, free CP is removed by magnetic separation, and HCR does not occur, thereby producing a low electrochemical signal.

DPV was adopted to demonstrate that HCR can enhance the current signal and can be achieved in this strategy. As shown in Figure 2, the electrical responses after HCR is higher than those before HCR. So it can be concluded that HCR can enhance the signal.

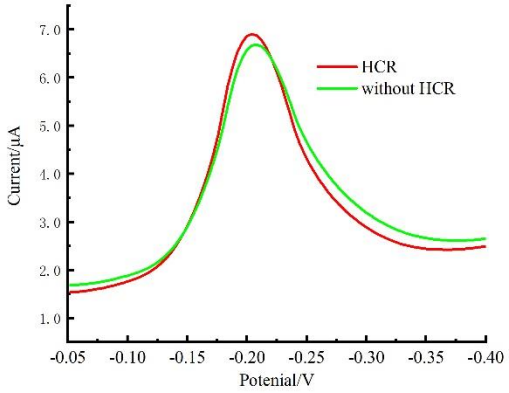
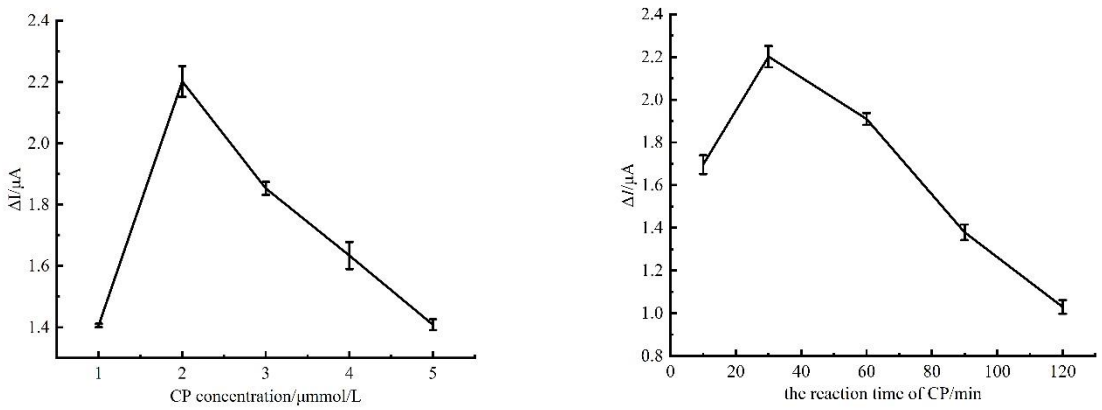


Figure 2. DPV responses with and without HCR in 0.1 mol/L PBS buffer solution (pH = 7.0) at kanamycin concentration of 1.00×10^{-12} mol/L.

3.2 Optimization of experimental conditions

The optimal electrochemical performance exhibits the sensitivity of this proposed electrochemical aptasensor. As a result, some factors such as CP concentration, hybridization time between CP and kanamycin aptamer, hairpin probes concentration, hybridization time of HCR, MB concentration and MB incubation time, PBS concentration and pH were carefully considered.

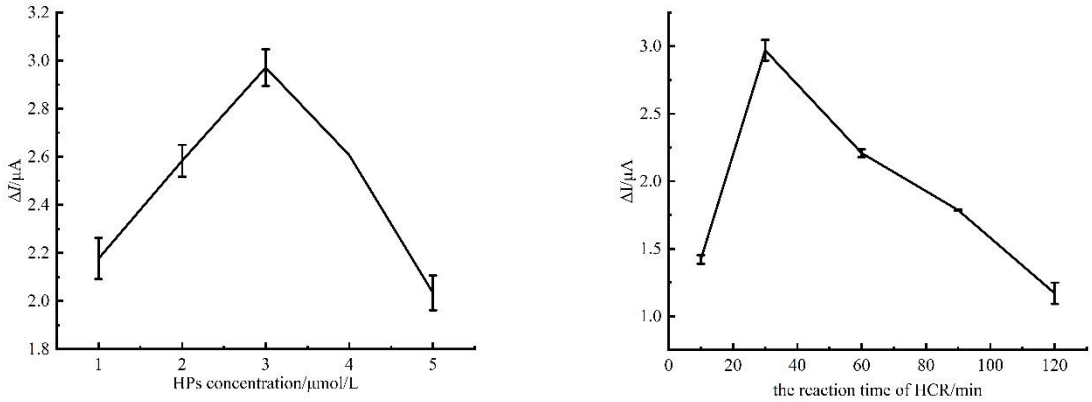
Given that CP is a trigger in HCR, the concentration and hybridization time of CP with kanamycin aptamer effect further the HCR and electrical signal. Therefore, CP concentrations from 1 μmol/L to 5 μmol/L and hybridization time from 10 min to 120 min were examined. As shown in Figure 3a, As shown in Figure 3a, ΔI increases at the concentration range of 1 ~ 2 μmol/L. When concentrations are higher than 2 μmol/L, the ΔI is inclined to decrease. Thus, 2 μmol/L CP was selected for further experiment. As shown in Figure 3b, ΔI increases first and then decreases from 30 min to 120 min, suggesting that a long hybridization time is not good for HCR. To save the assay time, 30 min is selected for hybridization time.



(a) (b)

Figure 3. (a) Effect of different CP concentrations on Δ*I*; (b) Effect of reaction time on Δ*I*.

The electrochemical signal is derived from the intercalated MB in the grooves of the formed dsDNA after HCR with H1 and H2. Accordingly, the amount of the formed dsDNA directly affects the performance and the length of this aptasensor. Figure 4 depicts the effect of hybridization time and hairpin concentration on the electrochemical signal. As shown in Figure 4a, with the concentration of HPs ranging from 1 μmol/L to 3 μmol/L, Δ*I* significantly increases, but further increases the HP concentration, and Δ*I* begins to decrease. The best Δ*I* was achieved when the concentration of HPs was 3 μmol/L, and this phenomenon is similar to that described in Ref. [26]. Thus, 3 μmol/L of HPs were selected to prepare the aptamer sensor. As shown in Figure 4b, Δ*I* initially increases with the increasing incubation time of HCR, and peaks at 30 min. A long incubation time does not raise the signal. Hence, 30 min is used as the optimum incubation time of HCR. In comparison with other HCR-based method [26] that spend 120 min for HCR, the proposed method is time-saving.

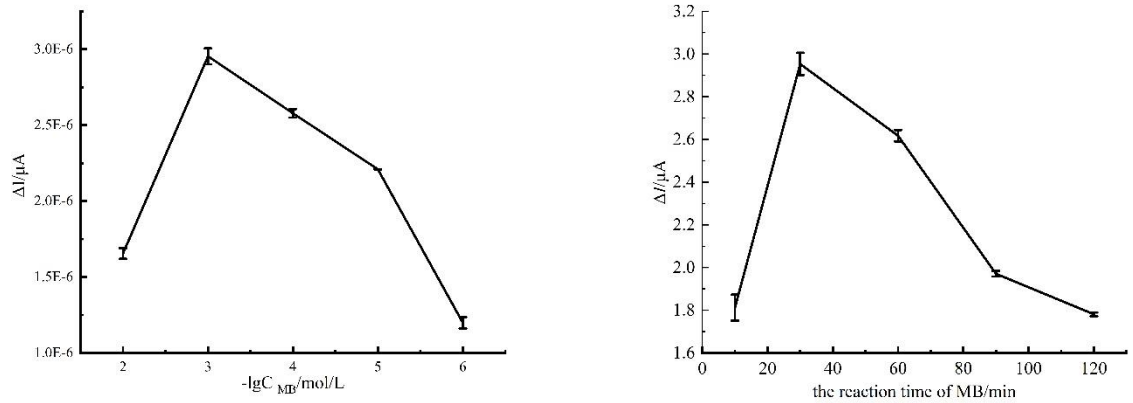


(a) (b)

Figure 4. (a) Effect of different hairpin probe concentration on Δ*I*. (b) Effect of reaction time on Δ*I*.

As an electroactive indicator, the amount of MB intercalated into the double-helix has a considerable effect on the electrochemical signal. The MB concentration and reaction time were

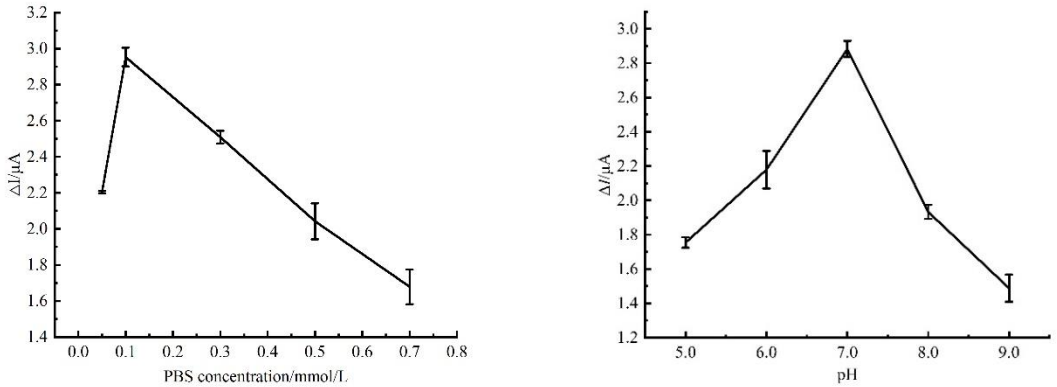
examined to ensure that MB absolutely embedded the grooves of dsDNA. As shown in Figure 5a, when MB concentration increases from 1.00×10^{-6} mol/L to 1.00×10^{-3} mol/L, ΔI increases remarkably, suggesting that MB is combined with dsDNA sufficiently. Further increasing MB concentration does not result in high ΔI. Therefore, 1.00×10^{-3} mol/L was chosen as the optimal concentration of MB. Figure 5b depicts effect of reaction time on ΔI. In the subsequent experiments, 30 min was used. The best ΔI was reached when reaction time was 30 min. These results were different with Ref [27] that the amount of MB was 5×10^{-6} mol/L and reaction time was 50 min. It was probably due to the reason that much more MB can make it absolutely intercalate into the dsDNA in a short time. Hence, 1.00×10^{-3} mol/L of MB and 30 min was used for subsequent experiment.



(a) (b)

Figure 5. (a)Effect of MB concentration on ΔI. (b) Effect of MB reaction time on ΔI.

Besides, the effects of PBS concentration and pH on ΔI were investigated. Results are displayed in Figure 6. The optimal PBS concentration is 0.01 mol/L, and the optimal pH value is 7.0. The results were similar to detection conditions that reported in Ref. [28].



(a) (b)

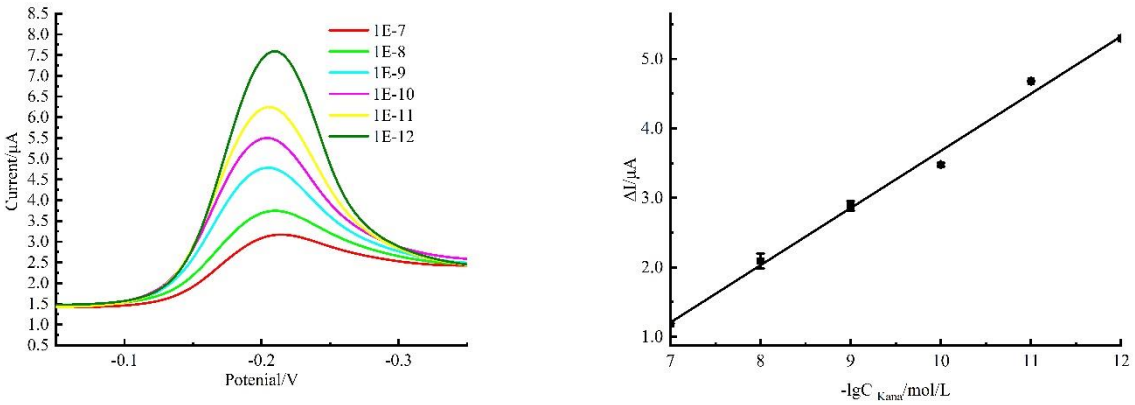
Figure 6. Effects of (a) PBS concentration on ΔI and (b) Effect of pH on ΔI.

3.3 Analytical performance of kanamycin assay

Under optimal experimental conditions, the proposed electrochemical aptasensor was adopted for the detection of kanamycin standards with different concentrations. As shown in Figure 7a, ΔI decreases with increasing kanamycin concentrations, suggesting that our strategy can be used to detect the target kanamycin. Figure 7b depicts a good linear relationship between ΔI and the logarithm of kanamycin concentration ranging from 1.00×10^{-12} mol/L to 1.00×10^{-7} mol/L. The linear regression equation is: ΔI (μA) =−0.824 lg$C$ − 4.560 (R^2 = 0.998) (where C is the kanamycin concentration). The detection limit (LOD) is 1.00×10^{-12} mol/L (S/N = 3).

The performance characteristics of other kanamycin detection methods are summarized in Table 2. The as-proposed electrochemical aptasensor can exhibit low LOD and a wide detection range in comparison with some of other detection methods. Some methods are superior than our strategy, however, there are some hindrances exist. For example, two methods described in Ref. [29] and [31] both need expensive enzymes (HRP or nicking enzyme) that is easy to deactivate. However, the proposed method was a non-enzyme type aptasensor, avoiding the high cost and low reproducibility from enzyme. In Ref. [29], [30], [31], [33], [34] and [35], the aptasensors were all labeled (biotin, MB, Cy3, FAM or hexaammineruthenium(III)). They need sophisticated label procedure and professional operation. By contrast, the proposed method was label-free, saving the modification procedure and lowering the cost. Besides, compared with the proposed method, the electrochemiluminescence aptasensor described in Ref. [31] needed a relatively complex detection platform.

The reproducibility of this electrochemical aptasensor was also investigated by detecting three high (1.00×10^{-7} mol/L), middle (1.00×10^{-9} mol/L), and low (1.00×10^{-11} mol/L) kanamycin concentrations. The relative standard deviations (RSDs) of intra-batch are 4.13%, 3.86% and 4.67% and the RSDs of inter-batch are 4.32%, 5.52% and 5.39%, showing that this aptasensor exhibits good reproducibility. Furthermore, the stability of the as-prepared aptasensor stored in 4 °C was monitored by analyzing 1.00×10^{-8} mol/L of kanamycin every two days for 14 days, the aptasensor retains 93.28% of its initial response, indicating an acceptable stability of the proposed method.



(a) (b)

Figure 7. (a) DPV responses of the aptasensor to different kanamycin concentrations. (b) Calibration curve of ΔI versus the logarithm of kanamycin concentration.

Table 2. Comparison of different kanamycin detection methods

Detection methods	Linear range (mol/L)	LOD (mol/L)	Reference
BA-ELISA	5.15×10^{-9}–5.15×10^{-7}	1.19×10^{-10}	[29]
Electrochemical	1.00×10^{-12}–1.00×10^{-6}	5.00×10^{-13}	[30]
Colorimetric	8.58×10^{-13}–3.43×10^{-11}	3.43×10^{-13}	[31]
Electrochemiluminescence	1.72×10^{-13}–1.70×10^{-7}	8.41×10^{-14}	[32]
SERS	1.72×10^{-7}–1.70×10^{-11}	1.55×10^{-11}	[33]
Fluorescence	1.00×10^{-10}–1.00×10^{-7}	1.0×10^{-10}	[34]
Photoelectrochemical	1.00×10^{-9}–3.00×10^{-7}	1.61×10^{-10}	[35]
Experiment	1.00×10^{-12}–1.00×10^{-7}	1.00×10^{-12}	-

3.4 Specificity of the electrochemical aptasensor

The specificity performance of the proposed aptasensor was evaluated by measuring kanamycin (1.00×10^{-9} mol/L) and four interfering substances (1.00×10^{-7} mol/L) including tetracycline, tobramycin, gentamicin and ampicillin. As shown in Figure 8, ΔI toward the kanamycin target is significantly higher than that toward the high-concentration non-target. This result suggests that the developed aptasensor has good specificity for the discrimination of the kanamycin target.

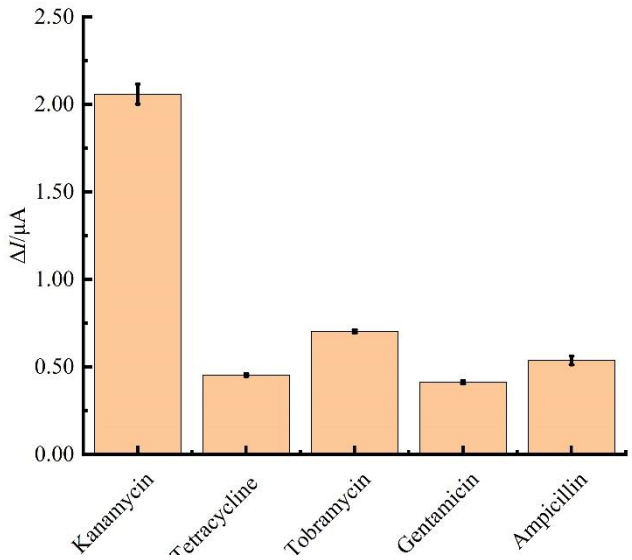


Figure 8. Specificity of proposed aptasensor in the presence of 1.00×10^{-9} mol/L kanamycin and 1.00×10^{-7} mol/L tetracycline, tobramycin, gentamicin, and ampicillin.

3.5 Application of this electrochemical aptasensor in milk samples

The standard addition method was used to analyze different kanamycin concentrations in milk samples to evaluate the feasibility of the present method for possible application. As displayed in Table

3, the recovery is between 97.75% and 101.50%, and RSDs range from 2.57% to 4.13%, clearly indicating that the proposed aptasensor is available for the kanamycin detection in milk samples.

Table 3. Milk recovery at standard rate

Number	Addition(mol/L)	Measured(mol/L)	RSD	Recovery
1	5.00×10^{-8}	4.96×10^{-8}	3.28%	99.13%
2	2.50×10^{-8}	2.52×10^{-8}	2.96%	100.80%
3	1.00×10^{-8}	9.94×10^{-9}	4.13%	99.42%
4	5.00×10^{-9}	4.92×10^{-9}	2.57%	98.34%
5	2.50×10^{-9}	2.51×10^{-9}	3.72%	100.30%
6	5.00×10^{-10}	5.08×10^{-10}	3.26%	101.50%
7	2.50×10^{-10}	2.44×10^{-10}	3.94%	97.75%

4. CONCLUSIONS

In summary, a novel electrochemical aptasensor for the sensitive, specific and label-free detection of kanamycin in milk was developed on the basis of the HCR signal amplification. MB intercalated into dsDNA could quantitatively reflect the change in kanamycin concentration. Under optimal conditions, the proposed aptasensor exhibited a good response ranging from1.00×10^{-12} mol/L to 1.00×10^{-7} mol/L, and the detection limit reached 1.00×10^{-12} mol/L. In comparison with other reported methods, our strategy displayed higher sensitivity and wider linear range. Besides, the proposed electrochemical aptasensor showed good selectivity, acceptable stability and reproducibility. The proposed aptasensor has been successfully applied in complex food samples, such as milk, and believed to be a promising platform for the rapid detection of antibiotics in food safety.

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