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# Aptamer biorecognition-triggered hairpin switch and nicking enzyme assisted signal amplification for ultrasensitive colorimetric bioassay of kanamycin in milk

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ABSTRACT

A colorimetric aptasensing strategy for detection of kanamycin was designed based on aptamer biorecognition and signal amplification assisted by nicking enzyme. The aptamer of kanamycin was designed to be contained in the metastable state hairpin DNA. The target DNA as recycling DNA was located in the loop of hairpin DNA. The presence of kanamycin stimulates the continuous actions, including specific recognition of the aptamer to kanamycin, the hybridization between target DNA and signal probe, the cleavage function of nicking enzyme. The actions induced accumulation of numerous free short sequences modified by platinum nanoparticles (PtNPs), which can catalyze the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB)-$H_2O_2$ to produce a colorimetric response. The aptasensor exhibited good selectivity and sensitivity for kanamycin in milk with a detection limit as low as 0.2 pg·mL$^{-1}$. In addition, the proposed assay is potentially to be extended for other antibiotics detection in foods by adapting the corresponding aptamer sequence.

## 1. Introduction

Kanamycin is one of the most important aminoglycoside antibiotics, which has been widely used for treating severe infections caused by Gram-positive and Gram-negative bacteria (Mohlenkamp & Anderso, 1968). It is only recommended for short-term use because abuse of this drug or animal-derived foods remaining this drug may result in ototoxicity, nephrotoxicity and antibiotic resistance (Economou & Gousia, 2015; Goldman, 2004; Hurd et al., 2008) To avoid overuse of kanamycin, the maximum residue limit of kanamycin in milk has been established by different countries, 150 μg/kg in the EU and 200 μg/kg in China (Deng et al., 2018; Yang et al., 2018). Therefore, in order to protect consumer and reduce the damage of excessive adverse effects, it is significant to develop reliable and rapid sensing platforms for quantitation of kanamycin for food security.

Traditional methods such as liquid chromatography coupled with mass spectrometry (Dijkstra et al., 2014; Mishra et al., 2017), high performance liquid chromatography (Chen et al., 2006; Hu et al., 2014), electrochemical methods (EC) (Wang et al., 2016; Zhao et al., 2011) and enzyme-linked immunosorbent assay (ELISA) (Kitagawa et al., 1983; Li et al., 2017) have been applied for the kanamycin detection. However, some imperfectness involved in the above detection techniques as time-consuming operation, the antibody of susceptible to environment restrict their application in food safety control. Aptamers as newly molecular recognition agents with high affinity to targets have attracted more attention of researchers (Gonzalez-Fernandez et al., 2013; Sosic et al., 2011; Zhao et al., 2015), and have been extensively utilized to build a sensing detection platform for kanamycin due to its advantages over high stability, easy modification and labeling (Robati et al., 2016; Zhu et al., 2012). A series of aptamer biosensors for kanamycin detection have been proposed with corresponding signal outputs, including fluorescence (Deng et al., 2018; Ramezani et al., 2016), colorimetry (Cui et al., 2018; Luan et al., 2017) and electrochemistry (Feng et al., 2019; Yao et al., 2019). Among different kinds of biosensors, the colorimetric aptasensors obtained more attention by means of low cost, efficiency, convenience, practicality and direct observation by the naked eyes (Song et al., 2011a, 2011b; Sun et al., 2014). However, how to improve the sensitivity by effective strategies is still a challenge for colorimetric assays. To achieve this purpose, different of signal amplification strategies with the assist of enzyme have been

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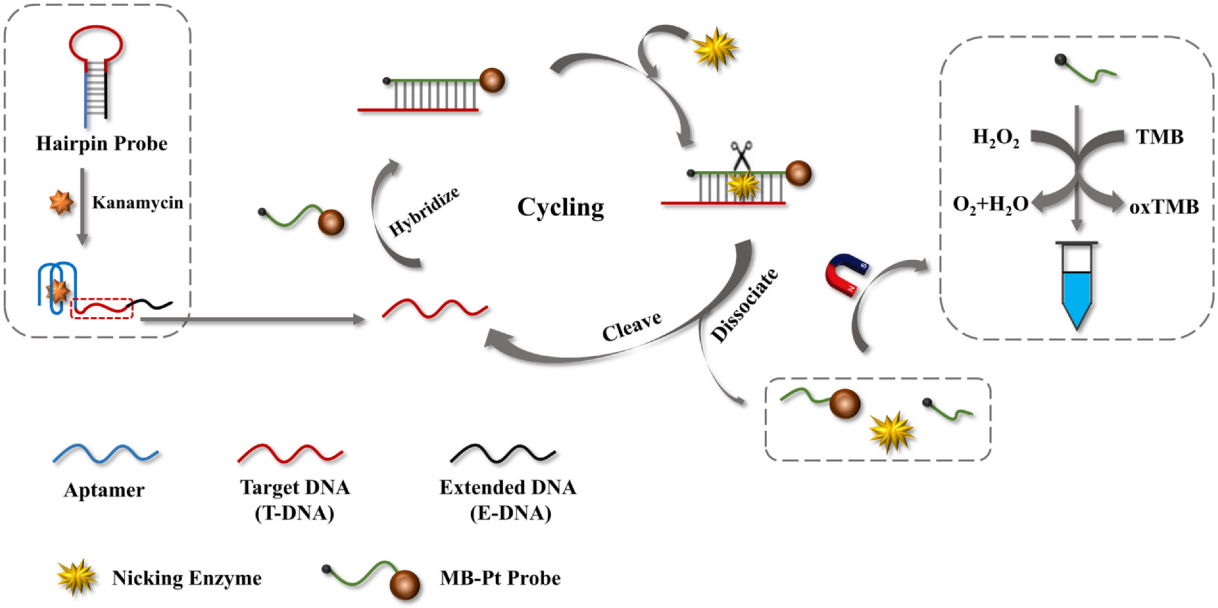


**Scheme 1.** Schematic illustration of colorimetric detection of kanamycin based on nicking enzyme assisted signal amplification.

introduced into colorimetric assays (Chen et al., 2019).

Inspired by the discovery of kanamycin aptamer and enzymatic signal amplification technique, some exonuclease-assisted aptasensing strategies have been developed for kanamycin detection (Chen et al., 2018; Ramezani et al., 2016). DNA nicking endonucleases as a special member of restriction endonucleases, is used to be an assistant strategy in sensitive colorimetric assays (Wang et al., 2016; Wu et al., 2018). Nicking enzyme signaling amplification reactions can be performed in mild isothermal condition without harsh condition and specialized instrumentation (Huang et al., 2013; Xue et al., 2012). As far as we know, there is no relevant literature reported about the development of nicking enzyme assisted signal amplification colorimetric aptasensor for kanamycin detection in real samples.

In this work, nicking enzyme-assisted DNA recycling strategy is applied to develop the colorimetric kanamycin aptasensor. The principle of the proposed method was shown in Scheme 1. The strategy takes the aptamer DNA contained in hairpin probe as the bio-recognition element, extended DNA as auxiliary stabilizer to the hairpin structure, target DNA located in the hairpin ring as recycling DNA which can be free in the presence of target, the difunctional-modified DNA as the signal probe and the separation tool for achieving better signal-to-noise ratio. In the presence of kanamycin, the formation of hairpin probe was induced from metastable hairpin shape to stretch form, thus prompting the hybridization between the target DNA and signal probe. Under the function of the nicking enzyme, the signal probe was cleaved; the PtNPs modified in signal probe were released. After magnetic separation, a significant blue color was obtained due to the reaction of TMB and $H_2O_2$ catalyzed by numerous PtNPs accumulated through continuous enzyme cleavage. The colorimetric signal is amplified through continuous enzyme cleavage. The proposed nicking enzyme-assisted amplification biosensing strategy provides a versatile platform for high sensitive and specific detection other antibiotics through adapting the target and corresponding aptamer sequence.

## 2. Experimental sections

### 2.1. Apparatus

The size and the morphology of PtNPs were performed on the transmission electron microscopy (TEM) using Tecnai G2 F20 Field Transmitance Electron Microscropy (FEI, Japan). The UV–vis spectra of the system were performed on Shimadzu Spectrophotometer UV-1800 (Shimadzu corporation, Kyoto, Japan). The wavelength of UV detector was determined at 455 nm and the injection volume was 200 μL.

### 2.2. Materials and reagents

Chloroplatinic acid ($H_2PtCl_6$), 3,3′,5,5′-Tetramethylbenzidine (TMB), kanamycin sulfate, gentamycin sulfate, tetracycline hydrochloride, streptomycin sulfate, chloramphenicol and tobramycin were purchased from Aladdin (Shanghai, China). Streptavidin modified magnetic beads (MBs) were obtained from the emerther company. (Jiaxing, China). Nicking endonuclease, Nb.BbvCl, and buffer were prepared by New England BioLabs. (Ipswich, MA, USA). The PBS buffer (137 mM NaCl ; 2.7 mM KCl ; 8 mM $Na_2HPO_4$ ; 1.8 mM $KH_2PO_4$ ; pH 7.4), hairpin probe (HP) and MB-Pt probe were supplied by Sangon Biotech Co., Ltd. (Shanghai, China). The HP is designed as follows: kanamycin aptamer (blue sequence: TGGG GGT TGA GGC TAA GCC GA), target-DNA (*T*-DNA, red sequence: CTT GCT CCT CAG CAA G) and extended-DNA (E-DNA, black sequence: TCG GCT TAG CC) and the other relevant oligonucleotide sequences used in the subsequent work were listed as Table S1. (HP1 matched with 8 base-pairs; HP2 matched with 11 base-pairs; HP3 matched with 14 base-pairs.) All other chemicals were of analytical reagent grade and used without further purification. Ultrapure water (18.25 MΩ cm) used throughout all experiments was prepared from a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

### 2.3. Preparation of the MB-Pt probe

PtNPs were synthesized according to the proposed method (Wang et al., 2017), the concrete procedure was shown in the supporting information. Then, the 100 μL PtNPs (1 mg·mL$^{-1}$) were incubated with 20 μL sulfydryl modified DNA (100 μM) for 24 h. The Pt-DNA probe was purified by centrifugation. The centrifugal supernatant was abandoned, precipitate was dissolved in PBS buffer. Then as-prepared Pt-DNA probe solution was incubated with 5 mg·mL$^{-1}$ streptavidin MBs at a ratio of 1:1 by shaking for 30 min at 37 °C. The MB-Pt probe to be successfully prepared was repeatedly washed with PBS buffer for more than 3 times using an external magnetic field, and the unbound Pt-DNA probe was removed. Resuspended magnetic separation probe in a final volume of 200 μL PBS and stored at 4 °C, and the MB-Pt probe solution was

obtained.

### 2.4. Colorimetric detection

Colorimetric detection of kanamycin was performed to confirm the extent of detectable concentration of kanamycin with the TMB–$H_2O_2$ chromogenic system. A series of kanamycin solutions (10 μL) were incubated with 2.5 μM of hairpin probe (15 μL) by shaking for 30 min at 37 °C. After that, the MB-Pt probe solution (15 μL) was added into and incubated for 1 h at 37 °C. Then 600 U·mL$^{-1}$ Nb.BbvCl (20 μL) was added and incubated for 1 h at 37 °C. After magnetic separation treatment, 50 μL of supernatant was extracted to mix with 50 μL of 1 mM TMB and 50 μL of 6 mM $H_2O_2$ (buffer: $C_6H_8O_7$ 16.7 mM; $CH_3COONa$ 0.33 M; pH 4.5) at 45 °C for 10 min in the dark. Then to terminate the reaction, 50 μL of 2 M $H_2SO_4$ was added into. Finally, the absorbance of the mixture was detected at 455 nm on UV-1800 spectrophotometer.

### 2.5. Treatment of real sample

To validate the effectiveness of the designed aptasensor, milk (cow milk and goat milk) was used as real food samples. The artificially contaminated milk was prepared by spiking a standard kanamycin solution into the real milk obtained from a local supermarket. Before detection, the contaminated milk samples were firstly pretreated by adding with 20% acetic acid dropwise to adjust the pH of solution to 4.6. After that, they were incubated in water bathed at 45 °C for 10 min to precipitate protein. Subsequently, the proteins and adipose were removed by centrifuging with 5000 rpm for 20 min. Finally, the filtrates obtained by filtration with a 0.22 μm membrane were adjusted to neutral pH and applied to the fluorescence assay

## 3. Results and discussion

### 3.1. Characterization of PtNPs

PtNPs were characterized by transmission electron microscopy (TEM). As shown in Fig. 1, TEM images exhibits the spherosome structure of PtNPs. The size of PtNPs is around 4 nm, and the dispersity is satisfactory. These results indicated that the particles are uniform and can be used in subsequent experiments.

### 3.2. Sensing principle

The designed strategy for the colorimetric detection of kanamycin is depicted in Scheme 1. The HP is designed as follows: kanamycin aptamer, target-DNA (*T*-DNA) and extended-DNA (E-DNA). Kanamycin aptamer (blue segment) is employed as the bio-recognition element, E-DNA (black segment) is used as assistant element to stabilize the hairpin structure of HP which is partly complementary to aptamer, *T*-DNA (red segment) is designed as recycling DNA which can be reused to capture MB-Pt probe in the presence of kanamycin. MB-Pt probe DNA with a specific enzyme cleavage site is designed as signal probe DNA which is respectively modified with MB at 5′ end and PtNPs at 3′end. In the presence of kanamycin, the specific binding between the target and aptamer can open the hairpin structures and make the *T*-DNA bind to the MB-Pt probe by hybridization reaction. After adding the nicking enzyme, MB-Pt probe is cleaved into two segments due to the enzyme cleavage, the double chain conformation is disintegrated and the whole HP combined with kanamycin is released again. Then, the *T*-DNA contained in the HP participated in the following cycles, leading to the fact that a great amount of short DNA labeled with PtNPs can be free from the MB-Pt probe. Finally, after magnetic separation, the free short DNA labeled with PtNPs in supernatant catalyzed TMB–$H_2O$ color reaction. In the absence of kanamycin, the supernatant only contains enzyme without short DNA labeled PtNPs, so the TMB–$H_2O_2$ reaction cannot be catalyzed. The results indicated that kanamycin can be quantitatively detected according to the change of light absorption value of the system.

### 3.3. Feasibility of the sensing strategy

In order to evaluate the feasibility of this strategy, the experimental group and negative control experiment were carried out first. Three curves are shown in Fig. 2 that respectively represented in the presence of: (I) for HP, signal probe DNA, it shows a negligible absorbance at 455 nm (curve a); (II) for HP, signal probe DNA and kanamycin, without the presence of enzyme, PtNPs with catalytic capacity cannot be free from the MB-Pt probe, it also shows a negligible absorbance (curve b); (III) for HP, signal probe DNA and enzyme, there is a low absorption peak appeared due to the background signal generated by the unstable hairpin structure HP forming the double chain with the MB-Pt probe (curve c); (IV) for condition (III) with the addition of kanamycin-the absorbance increased obviously (curve d), which is attributed to the following reactions, including the recognition and special binding between the aptamer and the kanamycin, the stretched of the target DNA, the formation of the double chain through hybridization reaction, subsequent enzyme digestion reaction, magnetic separation and the catalytic performance accomplished by the PtNPs in supernatant. In the light of the above results, significant desirable catalytic effect in TMB–$H_2O_2$ reaction induced by the PtNPs which was aggregated by addition of the target, it strongly demonstrated that the design of the strategy we proposed is successful.

### 3.4. Optimization of experimental conditions

To achieve the best assay performance of the experimental results, a series of optimization experiments were carried out. The length of E-DNA in our proposed assay is a key point of the experiment. When it is

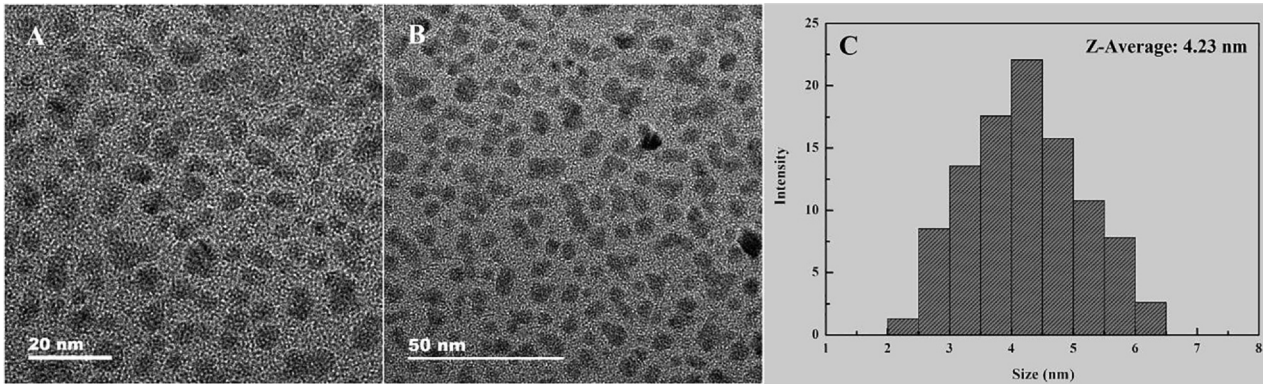


**Fig. 1.** (A, B) TEM images corresponding to the PtNPs. (C) Diameter distribution of PtNPs.

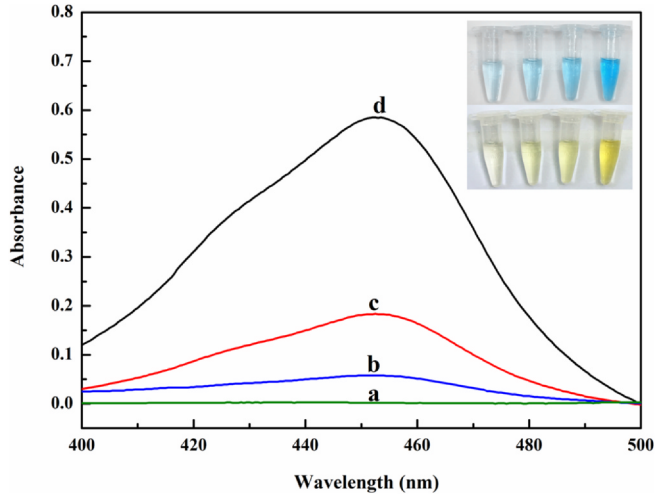


**Fig. 2.** UV–Vis spectra of TMB-$H_2O_2$ in the different conditions: (a) HP + signal probe DNA; (b) HP + signal probe DNA + kanamycin; (c) HP + signal probe DNA + Enzyme; (d) kanamycin + HP + signal probe DNA + Enzyme. Inset: colorimetric response of TMB-$H_2O_2$ in different conditions from left to right corresponding a to d.

too long, the binding capacity of aptamer and kanamycin is not enough to open the hairpin probe. When it is too short, the hairpin probe is not stable enough to ensure low background. Therefore, it is necessary to select a proper HP to initiate the subsequent reaction by changing the length of the stem. We compared colorimetric ΔA (A: express the presence of target; $A_0$: express the absence of target) of three different HPs. As shown in Fig. S1, E-DNA of different HP respectively matched kanamycin aptamer with 8 base-pairs, 11 base-pairs, 14 base-pairs. The highest ΔA was observed for HP with 11 bases. Thus, we chose the HP with 11 bases for next experiments.

We recorded the concentration of HP, incubation time of target DNA and MB-Pt probe, the enzyme concentration and the reaction time of enzyme digestion. As shown in Fig. S2, the highest ΔA was found at the the HP concentration 2.5 μM. As displayed in Fig. S3, the optimum incubation time of target DNA and MB-Pt probe was obtained at 60 min. The best response to kanamycin was obtained when the nicking enzyme concentration was 600 U·mL$^{-1}$. The effect of enzyme digestion reaction time was shown in Fig. S4 which reached to the plateau stage at 60 min.

### 3.5. Analytical performance of the sensing platform

The sensitivity of this sensor strategy was measured under optimized experimental conditions. Fig. 3 displays the colorimetric absorbance at 455 nm responded to the concentration of kanamycin. It could be seen from Fig. 3A, the absorbance increased linearly with the growing up kanamycin from 0.5 pg·mL$^{-1}$ to 200 ng·mL$^{-1}$. The correlation coefficient is 0.9945 and the detection limit is 0.2 pg·mL$^{-1}$ (S/N = 3). As shown in illustration photograph of Fig. 3A, the color change with various concentrations of kanamycin (0, 2, 10, 20, 100, 200 pg·mL$^{-1}$, respectively) would be distinguished by naked eye. The key performances of this method were compared with several methods reported in recent literature for determination of kanamycin in Table S2. It is clear that the detection limit comparable or superior to the other strategies for kanamycin detection. The low detection limit could be attributed to the large amount of DNA labeled with PtNPs, which can significantly catalyze the color reaction. The reproducibility of the developed aptasensor was assessed through five repeated measurements of 100 ng·mL$^{-1}$ of kanamycin and the relative standard deviation (RSD) was about 3.63%. The results also indicate that our developed aptasensor can be used to detect kanamycin sensitively and accurately.

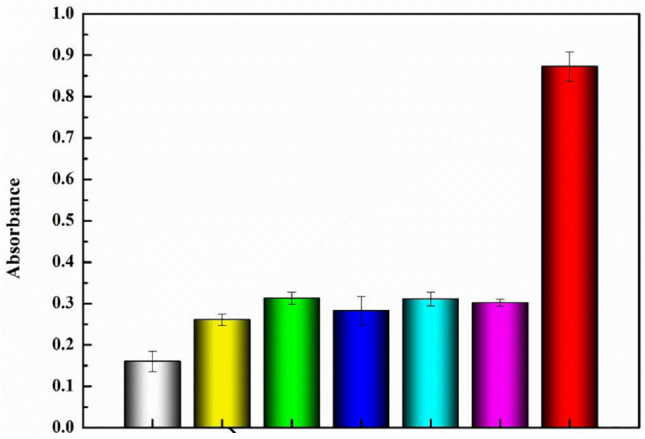


**Fig. 4.** Specificity and selectivity against other antibiotics.

### 3.6. Detection specificity and stability

Specificity is one vital feature in the establishment of successful antibiotic detection methods. The selectivity experiment was carried out by another five common antibiotics (gentamicin sulfate, tetracycline hydrochloride, streptomycin sulfate, chloramphenicol, and tobramycin) which were used as the negative controls to validate the specificity of the aptasensor. Fig. 4 shows only the presence of 0.2 μg·mL$^{-1}$ kanamycin, which was much lower than 2 μg·mL$^{-1}$ of

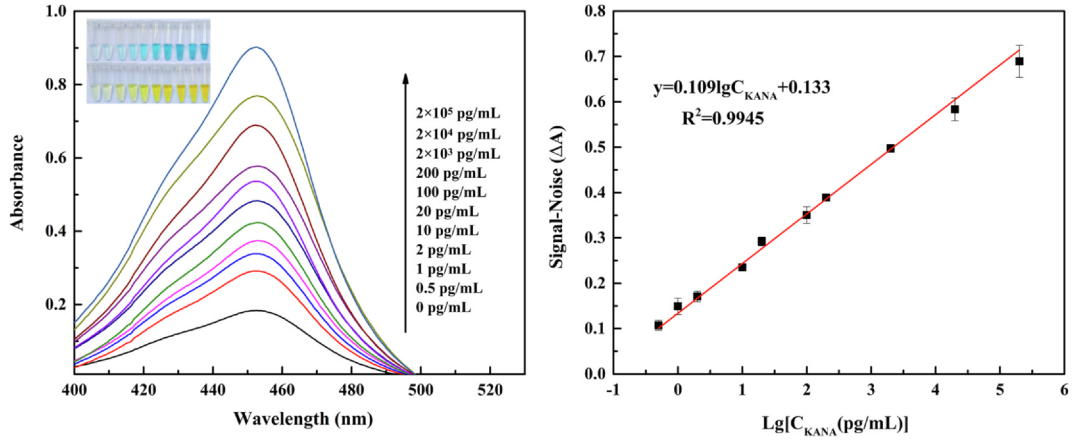


**Fig. 3.** (A) UV–vis spectra of TMB-$H_2O_2$ sensing system with different concentrations of kanamycin target and (Inset) colorimetric response to different concentrations of kanamycin (kanamycin: 0.5 pg·mL$^{-1}$ to 200 ng·mL$^{-1}$). (B) Linear curve of the relative absorbance in the range of 0.5 pg·mL$^{-1}$ to 200 ng·mL$^{-1}$.

**Table 1**
Detection of kanamycin in milk samples by our developed biosensor and ELISA.

| Sample | Spiked amount ($ng{\cdot}mL^{-1}$) | Measured (($ng{\cdot}mL^{-1}$, ± SD, n = 5) | | Recovery (%) | |
|---|---|---|---|---|---|
| | | Aptasensor | ELISA | Aptasensor | ELISA |
| Cow Milk | 0.5 | 0.476 ± 0.019 | 0.482 ± 0.017 | 95.3 | 96.4 |
| | 5.0 | 4.964 ± 0.182 | 5.115 ± 0.172 | 99.3 | 102.3 |
| | 10.0 | 10.227 ± 0.323 | 10.011 ± 0.215 | 102.3 | 100.1 |
| Goat Milk | 0.5 | 0.489 ± 0.022 | 0.499 ± 0.013 | 97.8 | 99.8 |
| | 5.0 | 5.123 ± 0.168 | 5.011 ± 0.193 | 102.5 | 100.2 |
| | 10.0 | 10.418 ± 0.385 | 10.363 ± 0.288 | 104.2 | 103.6 |

another five antibiotics, caused obvious differences in the absorbance. The result shows that the developed aptasensor for kanamycin has good specificity.

The stability of the developed aptasensor was assessed after the MB-Pt probe was stored at 4 °C for one month (Fig. S6). The absorbance was measured after incubating 1 $ng{\cdot}mL^{-1}$ kanamycin with the MB-Pt probe for different times. Compared with the newly prepared MB-Pt probe, the absorbance of the probe after storing for 30 days hardly decreased, and the signal intensity was close to 92.5% of its initial value (the absorbance of initial phase: 0.67, and the absorbance after one month: 0.62). As a consequence, the aptasensor indicated good stability.

### *3.7. Detection of kanamycin in milk samples*

To further evaluate the practical applicability of this sensor, the quantitative detection of kanamycin in spiked milk sample was carried out by a classic ELISA method and our method, respectively. The compared results of different methods are shown in Table 1. As can be seen from Table 1, different concentrations of kanamycin were added into milk, the measured data obtained using our method were in almost accordance with those obtained via the ELISA method. These results indicate that the developed aptasensor has reasonable accuracy and it is feasible to apply the aptasensor to analysis of real milk samples.

## 4. Conclusions

In conclusion, we have demonstrated a reliable colorimetric aptasensor for kanamycin detection with good sensitivity and selectivity. The nicking enzyme-assisted target DNA recycling initiated by the aptamer biorecognition of kanamycin provides great signal amplification to improve the sensitivity. The method exhibited a good sensitivity which is much lower than the maximum allowable levels in the European Union. To test the practical application of this aptasensor, objective experiments were performed on real samples and the recovery rates were satisfactory. The aptasensor can be extended to detect other antibiotics by altering the corresponding aptamer sequences. Therefore, we believe that this colorimetric aptasensor design may be proved to be useful in assessment of antibiotic residues for food safety monitoring.

## CRediT authorship contribution statement

**Mei Liu:** Conceptualization, Validation, Investigation, Resources, Writing - review & editing, Visualization, Project administration, Funding acquisition. **Zongqi Yang:** Methodology, Formal analysis, Data curation, Writing - original draft. **Baoxin Li:** Resources, Supervision. **Jianxiu Du:** Methodology, Investigation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128059.

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