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Label-free exonuclease I-assisted signal amplification colorimetric sensor for highly sensitive detection of kanamycin

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ABSTRACT

A label-free colorimetric method based on exonuclease I (Exo I)-assisted signal amplification with protamine as a medium was developed for analysis of kanamycin. In this study, a double-stranded DNA (dsDNA) probe was tailored by manipulating an aptamer and its complementary DNA (cDNA) ensuring detection of target with high selectivity and excellent sensitivity. Herein, protamine could not only combine with negatively charged gold nanoparticles but also interaction with polyanion DNA. Upon addition of target kanamycin, the target-aptamer complex was formed and the cDNA was released. Thus, both aptamer and cDNA could be digested by Exo I, and the captured kanamycin was liberated for triggering target recycling and signal amplification. Under optimized conditions, the proposed colorimetric method realized a low detection limit of 2.8×10^{-14} M along with a wide linear range plus excellent selectivity. Our strategy exhibited enormous potentials for fabricate various kinds of biosensors based on target-induced aptamer configuration changes.

1. Introduction

Aminoglycoside antibiotics have been widely utilized in the field of prevention and treatment of various diseases as well as promotion the animal growth (Qin et al., 2017; Wang, Wang, Wang, & Chen, 2017; Zhu, Chandra, Song, Ban, & Shim, 2012). As a common aminoglycoside antibiotic, kanamycin has been extensively used in the veterinary medicine to treat gram-positive and gram-negative infections due to its strong spectrum activity and low cost (Han et al., 2017; Li et al., 2017; Ma, Sun, Tu, Zhang, & Diao, 2017; Xu et al., 2015; Saratale et al., 2020). However, the incorrect/uncontrolled use of kanamycin may lead to its residues in animal derived food, which resulting in ultimate accumulation in human body (Long, Zhang, Yang, Zeng, & Jiang, 2015; Zhou, Zhang, & Tian, 2014) further causing serious side effects such as ototoxicity and nephrotoxicity. Owing to the great threats to human health (Abnous et al., 2017; Luo et al., 2015), maximum-residue levels (MRLS) for kanamycin has been established by European Union (150 μg kg^{-1} in milk, 100 μg kg^{-1} in meat, 600 μg kg^{-1} in liver and 2500 μg kg^{-1} in kidney) (Ma et al., 2017). To date, various approaches have been reported for determination of kanamycin in food products or biological samples, such as high performance liquid chromatography (Abu-Qare & Abou-Donia, 2001; Blanchaert, Poderós Jorge, Jankovics, Adams, & Van Schepdael, 2013), capillary electrophoresis (García-Ruiz et al., 2005; Hernández, Borrull, & Calull, 2003) gas chromatography (Berijani, Assadi, Anbia, Milani Hosseini, & Aghaee, 2006) and enzyme-linked immunosorbent assay (Chen et al., 2008; Yu et al., 2013). Although these methods have made great progress in detection of kanamycin, the requirement of time-consuming sample preparation, tedious operation procedures, high consumption of various reagents or sophisticated equipment has seriously limit their further applications. Therefore, it is urgent to develop simple, convenient and sensitive strategy for the determination of kanamycin in foodstuff.

Colorimetric technique has been paid close attention in chemical and biological sensing, because it could be easily visual judgment by naked

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eye observation, has simple operation process, and avoids the need of sophisticated equipment (Liu & Lu, 2006; Sabela, Balme, Bechelany, Janot, & Bisetty, 2017). Especially, gold nanoparticles (AuNPs) based colorimetric methods have earned tremendous momentum due to their excellent localized surface plasmon resonance and high extinction coefficient (Cao & Li, 2011; Shahdordizadeh et al., 2018). Based on the changes in the absorbance of AuNPs from dispersion to aggregation, and color changes from wine red to purple or blue, researchers have promoted AuNPs-based colorimetry for detection of various species, including metal ions (Liu et al., 2015), DNA (Yang, Jockusch, Vicens, Turro, & Tan, 2005) small molecules (Wang, Chen, Wang, & Tan, 2017) and proteins (Chen et al., 2014).

Compared to antibodies, aptamers exhibit outstanding merits, such as excellent stability, in vitro synthesis, easy of modification and fix, small size (Chen, Xiong, Yu, & Lai, 2019), low toxicity and low cost. Given their unique advantages, aptamers have been broadly used as recognition element to construct aptamer-based biosensors. In this case, numerous aptasensors have been developed in the fields of food safety, environmental monitoring and clinical diagnosis, based on colorimetric (Han et al., 2017), fluorescence (Xing et al., 2016), electrochemical (Zhang et al., 2019), chemiluminescence (Hao, Gu, Duan, Wu, & Wang, 2016) and surface plasmon resonance (Tu, Sun, & Grattan, 2014).

In order to obtain high sensitivity, different signal amplification techniques are used in aptamer-based colorimetric biosensors (Ramezani, Danesh, Lavaee, Abnous, & Taghdisi, 2016), such as hybrid chain reactions (HCR) (Blanchaert et al., 2013), polymerase chain reactions (PCR) (Xu, Ying, & Ping, 2019), tool enzymes (Ma et al., 2018) and roll ring amplification (RCA) (Liu et al., 2013). Among these signal amplification strategies, the signal amplification technology based on tool enzyme assisted has been rapidly developed in biochemical analysis in recent years due to its merits of simple operation, high sensitivity and specificity, as well as relatively short reaction time (Liu et al., 2013). Among these signal amplification strategies, nuclease-assisted signal amplification technology designed by combining the cycle effect of nuclease digestion, aptamer and nanotechnology has been applicated frequently in biochemical analysis due to its merits of simple operation, high sensitivity and specificity, as well as relatively short reaction time (Liu et al., 2013). There are two main types of signal amplification strategies based on nuclease assisted signal amplification (Chen et al., 2018; Zhang et al., 2019): one is the sequence dependent signal amplification strategy with restriction enzyme or restriction enzyme assisted signal amplification; the other is the signal amplification strategy without sequence dependent nuclease assisted signal amplification. In terms of the universality of nuclease-assisted signal amplification strategy, the exonuclease without sequence dependence is simpler and more feasible in the aspect of probe design and target analysis, thus which shows a broader application prospect.

Herein, in this study, a simple, sensitive and specific kanamycin colorimetric sensor was developed based on exonuclease I (Exo I)-assisted signal amplification and protamine mediated aggregation of AuNPs. The introduction of aptamer as recognition element ensures the specificity of this strategy owing to its strong affinity for the target molecule. In general, the target binds to the aptamer at a ratio of 1:1, which means one target can be recognized and bound by one aptamer. Fortunately, Exo I break this boundary which can specifically shear the single stranded cDNA as well as the kanamycin aptamer that bind to the target, destroying the structure of the target-aptamer complex, thus releasing the target to participate in the new binding cycle. In this case, the signal could be significantly amplified, and thus make the detection more sensitive and efficient. Positively charged protamine is a polycation rich in basic arginine residues, which can not only electrostatically combine with negatively charged DNA, but also interact with negatively charged AuNPs. And the interaction between protamine and AuNPs can induce the aggregation of AuNPs, thereby causing changes in the surface plasmon resonance (SPR) absorptions. Our Exo I-assisted signal amplification colorimetric assay only needs simple mixing of the dsDNA probe, target, protamine and AuNPs in homogeneous solution, without containing modification, or separation steps. By altering the aptamer sequence, it can be easily extended to detect other targets, which demonstrating that this method has a great potential as a valuable tool for screening multiple antibiotic residues in food safety.

2. Experimental section

2.1. Reagents and chemicals

Protamine sulfate salt, tetrachloroauric (III) acid tetrahydrate ($HAuCl_4{\cdot}4H_2O$), and sodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) were purchased from Sigma-Aldrich (USA). Kanamycin sulfate, tetracycline hydrochloride, p-hydroxy-ampicillin, chloramphenicol, ampicillin and gentamicin sulphate were obtained from Sangon Biotechnology Inc. (Shanghai, China). Kanamycin aptamer (5′-TGG GGG TTG AGG CTA AGC CGA-3′) and cDNA (5′-TCG GCT TAG CCT CAA-3′) were synthesized by Sangon Biotechnology Inc. (Shanghai, China). Sodium chloride (NaCl), sodium borohydride ($NaBH_4$), magnesium chloride ($MgCl_2$), glucose, and absolute ethyl alcohol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Exonuclease I was purchased from New England Biolabs (Beijing, China). All other chemicals were of analytical reagent grade or higher and used without further purification. Double distilled water (18.2 MΩ, Pall Cascada) was used throughout the experiments.

2.2. Instrumentation

SPR absorption spectra were recorded by using a Thermo Scientific NanoDrop 2000/2000C spectrophotometer (USA). The transmission electron microscopy (TEM) images were acquired using a JEM-1400 electron microscope (Japan) with an accelerating voltage of 100 kV. Dynamic light scattering (DLS) measurement was carried out with a Nano-ZS90 instrument (Malvern, UK).

2.3. Preparation of nanoparticles

AuNPs were prepared by the sodium citrate reduction of $HAuCl_4$ method with necessary modification (Liu & Lu, 2006). Prior to the synthesis experiment, all the glassware used was thoroughly soaked in aqua regia (3 parts HCl, 1 parts HNO_3), rinsed with ultrapure water, and oven-dried before using. Typically, 100 mL aqueous solution consisting of 1 mM $HAuCl_4$ was heated with vigorous stirring in a three-necked round-bottomed flask equipped with a reflux condenser. After boiling, 10 mL of 38.8 mM sodium citrate solution was added rapidly to the above solution. The color of the solution changed from yellow to light gray, and finally to wine red within 15 min. Finally, the resultant AuNPs was cooled to room temperature and stored at 4 °C for further use. The absorption spectra, TEM image and DLS data were used to determine the average size of AuNPs (Fig. 2, Fig. S1). The size of the prepared AuNP is estimated to be 13 ± 2 nm. AuNPs with different sizes (28 nm, 31 nm and 42 nm) could be obtained by adjusting the volumes of 38.8 mM trisodium citrate, including 8, 6 and 4 mL, respectively (Figs. S2 and S3). And 8 nm AuNPs was prepared by the reduction of $HAuCl_4$ with $NaBH_4$ at room temperature following a previous method with slight modification. (Obare, Hollowell, & Murphy, 2002)

2.4. Colorimetric determination of kanamycin

In this experiment, the kanamycin-aptamer binding buffer was 10 mM HEPES buffer solution (pH 7.4) containing 50 mM NaCl and 2.5 mM $MgCl_2$. The various concentrations of standard kanamycin solution and aptamer solution were prepared with binding buffer.

For kanamycin analysis, the aptamer/cDNA double-stranded structure (dsDNA) probe was prepared according to the following procedure: 10 μL of 10 μM kanamycin aptamer and 10 μL of 10 μM cDNA were

initially mixed with binding buffer. Then, the resulting mixture was heated to 95 °C for 5 min and finally slowly cooled to room temperature within 2 h. Next, 5 μL of dsDNA probe (1.5 μM) and 5 μL of Exo I (1500 U mL^{-1}) were added into 40 μL kanamycin standards with different concentrations. The mixture was incubated for 1.5 h to execute Exo I-assisted target recycling. Subsequently, 100 μL of protamine (1.5 μg mL^{-1}) was added to the above solution and incubated for 10 min. Finally, 400 μL AuNPs was added to the above solution and incubated for another 2 min. The SPR absorption spectra of the mixture were recorded for quantitative analysis.

2.5. Detection of kanamycin in milk

Milk samples were obtained from local supermarkets. Milk samples are processed according to the method described by Zhang and Zhou (Zhou et al., 2014). Firstly, the acetic acid solution (20%, V/V) was drop wised to the milk sample until the pH was adjusted to 4.6, so that the protein in the milk (mainly tyrosine) was denatured and precipitated at the isoelectric point. Then, the sample was placed in a water bath at 45 °C for 10 min to ensure that the protein was completely precipitated. After that, the sample was centrifuged at 9580 × *g* for 30 min, followed by the removal of the precipitate containing denatured protein and fat. Subsequently, the supernatant was filtered through a 0.22 μm ultrafiltration membrane to remove lipids and the filtrates were regulated to neutral pH for further detection. Add a certain amount of kanamycin to the pre-treated milk sample, and then perform the kanamycin colorimetric detection according to the above steps.

3. Results and discussion

3.1. Design principle of detection kanamycin based on aptamer structure switch colorimetric sensor using Exo I as a signal amplifier

The construction of target-binding induced aptamer structure switch colorimetric sensor was auxiliary with the strategy of Exo I-assisted signal amplification and polycationic protamine mediated aggregation of negatively charged citric-coated AuNPs. The sensing mechanism was shown in Fig. 1. In our design, the electrostatic interaction between protamine and DNA is strongly limited by the length of the DNA strand. Exo I-assisted signal amplification was initiated due to the introduction of target kanamycin. Exo I is a kind of enzyme that specifically hydrolyzes phosphodiester bond from 3′ to 5′ in sequence, and the final product is a single nucleotide. In the presence of kanamycin, kanamycin specifically bound to the kanamycin aptamer, followed by dissociation with cDNA to form single strand that provided catalytic substrate for Exo I. In this case, the single stranded kanamycin aptamer and cDNA were hydrolyzed into small fragments by Exo I, accompanying failing to interact with protamine owing to the relatively weak electrostatic interaction. And the released target could be recycled during the cycle of bonding-hydrolysis-release. Together with the occurrence of target-binding induced aptamer structure switch and the Exo I-assisted signal amplification, the AuNPs could interaction with the remaining free protamine, which resulting in the aggregation of AuNPs with a color change from red to blue (Fig. 2). However, in the absence of kanamycin,

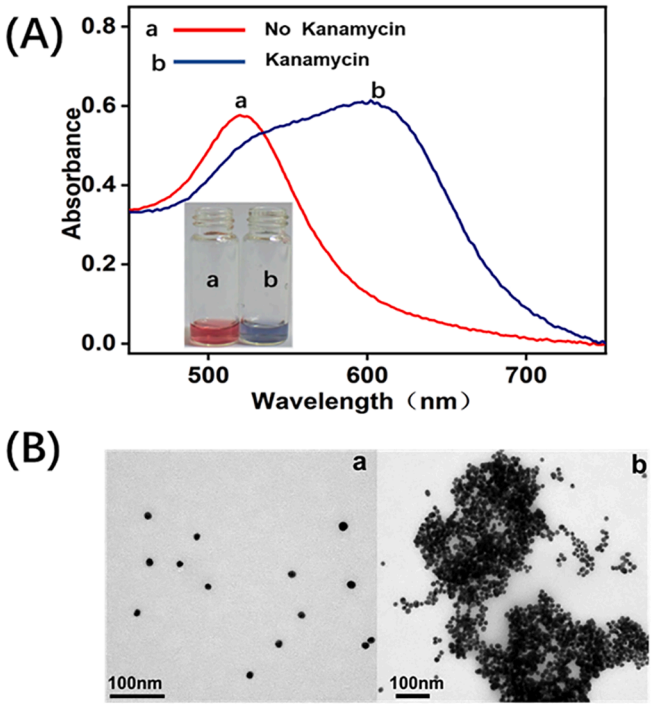

Fig. 2. (A) Absorption spectra and (B) corresponding TEM images of AuNPs in the absence (a) and presence (b) of kanamycin.

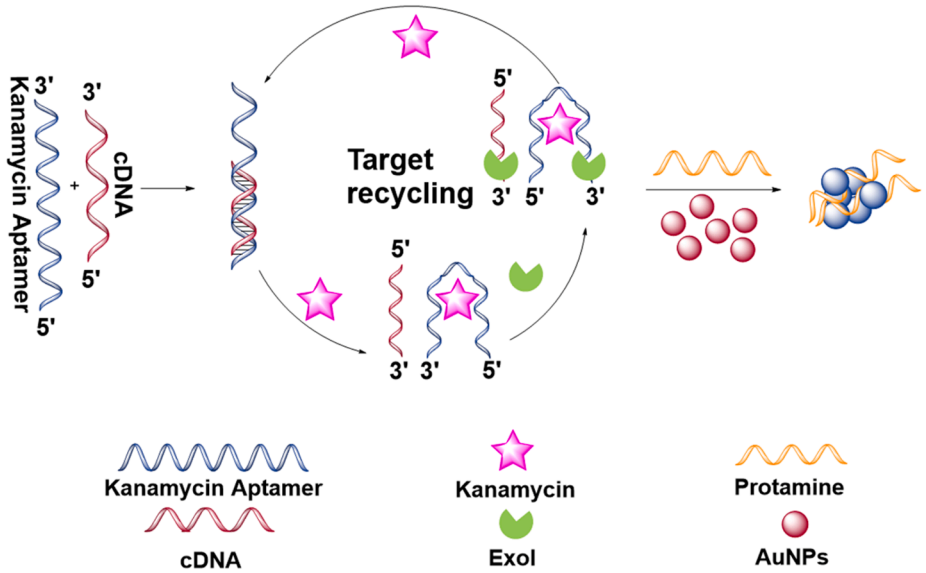

Fig. 1. Principle of the label-free Exo I-assisted signal amplification colorimetric sensor for highly sensitive detection of kanamycin.

the aptamer-cDNA dsDNA probe could preferentially interact with protamine, thus the AuNPs existed in a dispersed state with native color. Finally, the quantitative analysis of kanamycin could be achieved by measuring the change of SPR absorption of AuNPs.

3.2. Electrophoresis characterization

Agarose gel electrophoresis was employed to verify the catalytic activity of Exo I toward anti-kanamycin aptamer upon binding kanamycin. As shown in Fig. S4, lanes 1, 2 and 4 presented a bright band, whereas the lane 3, owing to the hydrolysis of the anti-kanamycin aptamer and/or cDNA by Exo I and then migrated out of the gel. This result confirms that the kanamycin aptamer can be cleaved upon binding kanamycin, which is consistent with the previous studies (Chen et al., 2018; Zeng, Tang, Zhang, Luo, & Tang, 2018).

3.3. Optimization of experimental conditions

The concentration of protamine severely restricted the degree of aggregation of AuNPs and the amount of dsDNA probes. Therefore, the effect of protamine concentration on the analytical performance was first optimized. As shown in Fig. S5A, the ratio of SPR absorption value at 650 nm to 520 nm (A_{650}/A_{520}) increased with the increase of the protamine concentration up to 1.5 μg mL^{-1}, while higher concentration of protamine did not obvious increased the ratio of SPR absorption. This occurred because the aggregation degree of AuNPs increased with the increase of the protamine concentration lower than 1.5 μg mL^{-1}, and further increasing the concentration, the aggregation degree was close to the maximum. Consequently, 1.5 μg mL^{-1} was chosen as the optimum protamine concentration.

In addition, the concentration of dsDNA probe was explored for improving the sensitivity. As revealed in Fig. S5B, the higher the concentration of dsDNA probe in the range of 0 to 2 μM, the smaller the ratio of A_{650}/A_{520} was obtained. This may be due to the fact that with the increase of dsDNA concentration, more and more dsDNA was combined with protamine, which leads to the gradually recovery of AuNPs to its original dispersed state. However, by further increasing the concentration of dsDNA, the ratio A_{650}/A_{520} is almost the same as that in the presence of 1.5 μM dsDNA. Therefore, the optimal concentration of dsDNA probe is 1.5 μM.

Next, the influence of the amount of Exo I on the signal amplification for Exo I-assisted target recycling was studied with five different concentrations. As illustrated in Fig. S6, it could be seen that the ratio of A_{650}/A_{520} of AuNPs gradually increased with increasing the concentration of Exo I owing to the enhanced signal amplification. When the concentration reached 1500 U mL^{-1}, our strategy displayed almost the highest ratio value. Thus, 1500 U mL^{-1} was chosen as the optimal concentration of Exo I.

Finally, the effect of the size of AuNPs was investigated. Five different diameters of AuNPs were tested including 8 nm, 13 nm, 28 nm, 31 nm and 42 nm. As shown in Fig. S7, the smaller the diameter of AuNPs in the range of 13–42 nm, the higher the sensitivity of our colorimetric sensor was for analysis of kanamycin. However, further decreasing the diameter of AuNPs would induce lower sensitivity. Thus, the diameter of 13 nm was chosen.

3.4. Sensitivity and selectivity for the detection of kanamycin

To assess the sensitivity, our proposed Exo I-assisted signal amplification strategy was applied for quantitative analysis of kanamycin under optimal conditions. As shown in Fig. 3A, with the increase of kanamycin concentration, the SPR absorption band of AuNPs at 520 nm region decreased while it increased at 650 nm. At the same time, the color change of AuNPs from red to blue was displayed, as can be seen in Fig. 3A. In this case, the two SPR absorption values at 520 nm and 650 nm were used to represent the relative amounts of dispersed and

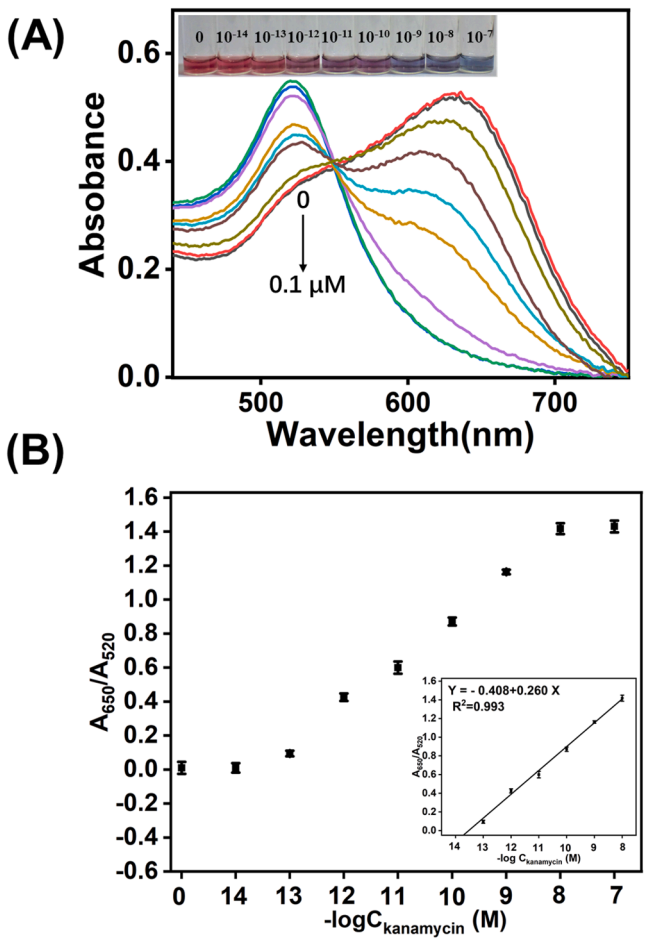

Fig. 3. (A) Absorption spectra (inset photographic images were the corresponding colorimetric response) of AuNPs in the system upon incubation with different concentrations of kanamycin (from left to right: 0, 10^{-14}, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M, respectively). (B) The plot of A_{650}/A_{520} values *versus* the concentrations of kanamycin in 10 mM HEPES (pH 7.4) buffer solution. Inset: the corresponding calibration curve of the Exo I-assisted system for detection of kanamycin. Error bars indicate standard deviations of five measurements.

aggregated AuNPs. Fig. 3B showed that the absorption ratio is linear in the range of 10^{-13} to 10^{-8} M in the concentration range of kanamycin. The limit of detection (LOD) value was calculated to be 2.8×10^{-14} M according to the $3\sigma/s$ method (LOD = $3\sigma/s$, σ is the standard deviation of the blank sample, and s is the slope of the standard curve), which is at least 1 orders of magnitude lower than that of reported colorimetry methods as well as other detection methods (Table S1) (Blanchaert et al., 2013; Chen et al., 2018; Han et al., 2019; Hao et al., 2016; Ramezani et al., 2016; Saratale et al., 2020; Xu et al., 2015; Yu et al., 2013).

To evaluate the selectivity of the developed strategy for kanamycin detection, the possible interfering agents including glucose, p-hydroxy-ampicillin, chloramphenicol, tetracycline hydrochloride, ampicillin and gentamicin sulphate were selected to test. As shown in Fig. 4, glucose, p-hydroxy-ampicillin, chloramphenicol, tetracycline hydrochloride, ampicillin and gentamicin sulphate did not cause obvious absorption ratio value increase. Only in the presence of kanamycin, the absorption ratio value was significantly enhanced, which indicated the strong interaction between kanamycin and the aptamer. These results demonstrated that our developed strategy provided attractive specificity toward kanamycin.

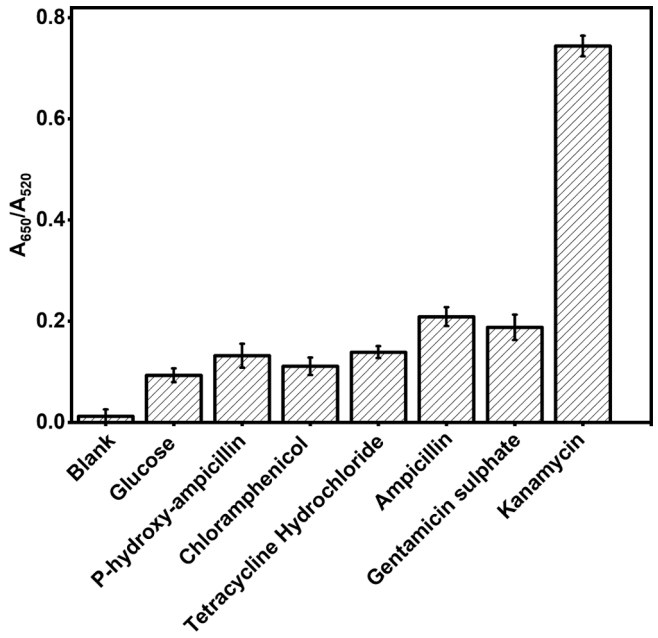

Fig. 4. Selectivity assay of the Exo I-assisted system upon the addition of kanamycin (10^{-10} M) and various potentially coexisting species (10^{-7} M). The error bars represent standard deviations from five parallel measurements.

3.5. Application to real milk samples

In order to further evaluate the potential application of the proposed atpasensor in real samples, milk samples were spiked with various concentrations of kanamycin. As shown in Fig. S8, the LOD for kanamycin was 5.5×10^{-14} M in milk samples with a wide dynamic linear range from 10^{-13} to 10^{-8} M, which is in agreement with that of the standard solutions. These results confirmed that our Exo I-assisted signal amplification assay is a very promising innovative tool in the accurate analysis of antibiotics-based screening detection due to its outstanding analytical sensitivity.

4. Conclusions

In this study, we have successfully developed a label-free target-binding induced aptamer structure switch colorimetric sensor for ultrasensitive detection of kanamycin based on Exo I-assisted signal amplification and polycationic protamine mediated aggregation of negatively charged AuNPs. This design took the full advantages of the outstanding signal amplification effect of Exo I, high binding affinity of aptamer with target as well as strong electrostatic interaction between protamine and negatively charged AuNPs, and thus successfully attained the ultrasensitive detection of kanamycin with detection limit of 2.8×10^{-14} M. Our aptamer colorimetric assay only needs simple mixing of the dsDNA probe, target, protamine and AuNPs in homogeneous solution, without containing modification, or separation steps. Besides, the proposed strategy could be applied for detection of kanamycin in milk samples with high reliability. Due to these advantages, this method is promising for wide applications to analysis other small molecules and proteins.

CRediT authorship contribution statement

Jingwen Li: Conceptualization, Methodology, Investigation, Writing - original draft. **Yongming Liu:** Investigation, Writing - review & editing. **Hao Lin:** Data curation, Validation. **Yan Chen:** Validation, Writing - review & editing. **Zhenbo Liu:** Data curation. **Xuming Zhuang:** Investigation. **Chunyuan Tian:** Validation. **Xiuli Fu:** Supervision, Funding acquisition, Writing - review & editing. **: . Lingxin Chen:** Funding acquisition, Project administration, Supervision, Conceptualization, Writing - review & editing.

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.2020.128988.

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