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Selection and identification of streptomycin-specific single-stranded DNA aptamers and the application in the detection of streptomycin in honey

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ABSTRACT

Single-stranded DNA (ssDNA) aptamers specific to streptomycin were screened and identified from a random oligonucleotides library by affinity magnetic beads-based SELEX. After eight rounds of selection, 16 ssDNA with different sequences were identified. Then the dissociation constants (K_d) of these ssDNA were determined and an aptamer (STR1) with highest affinity for streptomycin was identified. Further study showed that aptamer STR1 exhibits very low affinity for other aminoglycoside antibiotics, indicating high specificity. With this aptamer, detection of streptomycin was achieved by using gold nanoparticles (AuNPs)-based colorimetric method. In the presence of streptomycin, the competitive binding of the target and the aptamer decreases the stability of AuNPs in NaCl solution, triggers the aggregation, and exhibits visible color change of AuNPs solution. Through UV–visible spectroscopic quantitative analysis, streptomycin can be detected in the range of 0.2–1.2 μM. The presence of other aminoglycoside antibiotics shows neglectable disturbance. Furthermore, the established method was utilized to detect streptomycin in honey, and the same low detection limit and linear detection range were achieved.

1. Introduction

Streptomycin is an aminoglycoside antibiotic produced by *Streptomyces griseus*. Since its particular inhibition activity against gram-negative bacteria, streptomycin has been widely used not only as an antibacterial drug in human therapy, but also as a veterinary drug in animal husbandry, a crop-protection agents in agriculture, or for the treatment of bacterial honeybee disease in apiculture [1,2]. However, serious side effects of streptomycin have been well-reported, including allergic reactions, loss of hearing and toxicity to the kidneys, etc. [3–5]. When it is over-committed, streptomycin residue will exist in food products such as meat, milk and honey, which can be hazard for the health. Therefore, the European Commission established the maximum residue limits (MRLs) of 200 μg/kg for milk, 1000 μg/kg for porcine kidney and 500 μg/kg for porcine muscle [6]. Although no MRLs have been fixed for bee products in the EU, the use of streptomycin in natural products like honey is banned in many countries [1,7]. To establish efficient, accurate and economical methods for the detection of streptomycin residue is of great importance in food safety control.

A variety of methods have been reported for the determination of streptomycin residue in food matrices. Among them, high-performance liquid chromatography (HPLC) is a high-sensitive means which can provide reliable results. However, owing to the lack of chromophore group, post-column derivatization and fluorescence detection are required for trace level detection [8,9]. Liquid chromatography–mass spectrometry (LC–MS) was also employed for the detection of streptomycin and other antibiotics with excellent performance [10,11], while the complicated sample preparation and high cost restrict its applications. Immunochemical assays are attractive methods for the detection of low concentration of targets with high specificity and sensitivity. Various immunoassays, such as radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA), fluorescence immunoassay (FIA) and immunochromatographic assay (ICA) have been employed for the detection of antibiotics residues [12–16]. However, due to the cross-reactions with complicated compounds in food such as honey, immunoassays are susceptible to be interfered easily in real sample analysis [17]. Meanwhile, the production of antibodies against small molecular antibiotics is time-consuming and costly.

Aptamers are mainly oligonucleotides (ssDNA or RNA) that have been selected *in vitro* to combine with their targets with high affinity and specificity. Since the concept of aptamer was raised by Ellington and Szostak [18] and Bock [19], and the

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development of the technology named systematic evolution of ligands by exponential enrichment (SELEX) [20], a variety of aptamers against different targets, such as proteins, small molecules, ions, even cells have been reported. Compared with antibodies, aptamers have excellent stability as well as high affinity and specificity. Moreover, aptamers are easy to synthesize, modify, manipulate and characterize [21,22]. Therefore, numerous aptamer-based bioanalysis strategies have been proposed and applied in the detection of different molecules. Recently, aptamer-based sensors have been used to detect antibiotics, such as neomycin [23], kanamycin [24], tobramycin [25–27], tetracycline [28], oxytetracycline [29], ampicillin [30], etc. However, the reported aptamers specific to antibiotics are still very limited. Aptamers with excellent stability and specificity are of great significance for their wide applications in the detection of antibiotics residues.

Streptomycin and other aminoglycoside antibiotics have affinity for RNA molecules, because their natural targets are ribosomal RNA. However, RNA is usually lack of stability. Thus their applications in bioassays as recognition elements are restricted. ssDNA are comparatively stable. Whereas ssDNA aptamers specific to streptomycin have not yet been reported. Here we describe the selection and identification of ssDNA aptamers against streptomycin through affinity magnetic beads-based SELEX. The affinity and specificity of the screened aptamers were evaluated. Among them, aptamer STR1 exhibits high affinity for streptomycin and very low affinity for other aminoglycoside antibiotics. Therefore, STR1 was chosen for further development of aptamer-based streptomycin assay.

Owing to the unique physicochemical and excellent biocompatibility, gold nanoparticles (AuNPs) have been widely used in bioassays. Particularly, the change in color of AuNPs solution, which is accompanied with the aggregation of AuNPs, obtained extensive applications in the fabrication of colorimetric biosensors, which were utilized in the detection of enzyme activities [31], target nucleic acids [32,33], DNA-binding molecules [34–36], other small molecules [37] and ions [38–40], etc. In this study, a label-free AuNPs-based colorimetric assay was employed for the detection of streptomycin. Furthermore, the method was adopted to detect streptomycin in honey samples. The results show that the selected aptamer and the proposed detection method are highly specific to streptomycin, and rapid and sensitive detection of the target can be achieved both in the standard solution and in food samples.

2. Experimental

2.1. Chemicals and materials

Streptomycin sulfate, neomycin sulfate hydrate, kanamycin sulfate, gentamicin sulfate, tetracycline hydrochloride, ampicillin, 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sangon Biotech Co. Ltd (Shanghai, China). Dynabeads M-280 Streptavidin was obtained from Invitrogen Company. Epoxy magnetic beads were from Tianjin Chemical Regent Company (Tianjin, China). Reagents for polymerase chain reaction (PCR) amplification (*pfu*-polymerase and deoxynucleoside triphosphate [dNTP]) were purchased from TaKaRa Bio Inc (Dalian, China). Horseradish peroxidase (HRP)-conjugated streptavidin was procured from EarthOx Company. Honey was from local market.

A synthetic ssDNA library was selected as initial pool, which includes a random sequence of 35 nucleotides flanked by two primers binding sequences for PCR amplification and cloning (5′-TAGGGAATTCGTCGACGGATCC-N_{35}-CTGCAGGTCGACGCATGCGCC-G-3′). A forward primer (5′-TAGGGAATTCGTCGACGGAT-3′) or a biotinylated forward primer (5′-biotin-TAGGGAATTCGTCGACG-GAT-3′), and a reverse primer (5′-CGGCGCATGCGTCGACCTG-3′) or a biotinylated reverse primer (5′-biotin-CGGCGCATGCGTCGACCT-G-3′) were used for PCR amplification and ssDNA generation. All of the oligonucleotides were synthesized by Sangon Biotech Co. Ltd (Shanghai, China).

All other chemicals were of analytical grade. Ultrapure water (18.2 MΩ cm) was employed to prepare all aqueous solutions.

2.2. Modification of epoxy magnetic beads

Epoxy magnetic beads were used for covalent immobilization of streptomycin or other antibiotics. Briefly, 0.5 mg epoxy magnetic beads were washed three times with 0.1 M phosphate buffered saline (PBS, pH 8.0) and suspended in 200 μL PBS. Then 10 mM streptomycin was added to the beads. The mixture was incubated overnight at 37 °C with mild shaking. The amino groups (NH_2) of streptomycin were covalently coupled with the epoxy groups on the surface of the magnetic beads. The streptomycin-coated beads were washed three times with PBS and the free epoxy groups on the surface of the beads were blocked with 0.5 M ethanolamine (pH 8.0) for 6 h at 37 °C with mild shaking. Finally, the streptomycin-coated beads were washed and resuspended in 200 μL PBS, stored at 4 °C until use. To evaluate the coverage of streptomycin on beads surface, the unbound streptomycin was pooled and estimated by malt phenol colorimetric method [41].

Epoxy magnetic beads coated with ethanolamine were used for negative selection. To prepare ethanolamine-coated beads, 0.5 M ethanolamine (pH 8.0) was directly added to the washed epoxy magnetic beads. The mixture was incubated overnight at 37 °C with mild shaking. Finally, the ethanolamine-coated beads were washed and resuspended in 200 μL PBS, stored at 4 °C until use [42].

To evaluate the specificity of the selected aptamers, neomycin, kanamycin, gentamicin and tetracycline were immobilized onto the surface of epoxy magnetic beads, respectively, by using the same conditions for immobilization of streptomycin. The modified beads were then blocked with 0.5 M ethanolamine (pH 8.0).

2.3. In vitro selection

Several rounds of *in vitro* selection were carried out to screen aptamers specific to streptomycin. In the first round, streptomycin-coated magnetic beads were firstly washed with binding buffer (20 mM Tris–HCl containing 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, and 0.02% Tween 20, pH 7.6) for three times. 200 μL of 0.5 μM initial random ssDNA in binding buffer was incubated at 90 °C for 10 min, then cooled at 4 °C immediately for 15 min. 0.5 mg streptomycin-coated magnetic beads were added to ssDNA solution quickly and incubated for 30 min at room temperature with mild shaking. The beads were washed with binding buffer for five times to remove unbound oligonucleotides completely. In order to recover the adsorbed oligonucleotides from streptomycin-coated magnetic beads, the beads-DNA complex was incubated in 150 μL elution buffer (40 mM Tris–HCl containing 10 mM EDTA, 3.5 M urea, 0.02% Tween 20, pH 8.0) at 80 °C for 10 min with mild shaking. The process was repeated three times to elute all adsorbed ssDNA.

The eluted ssDNA were precipitated by ethanol, dissolved in 10 μL sterile water, and then amplified by PCR. Each 50 μL of PCR reaction mixture contained 4 μL of dNTP (2.5 mM), 1 μL forward primer (20 μM), 1 μL biotinylated reverse primer (20 μM), 0.25 μL Taq DNA polymerase (2.5 U), 10 μL template ssDNA and 5 μL PCR buffer (100 mM Tris–HCl buffer containing 500 mM KCl and 15 mM $MgCl_2$, pH8.3). PCR amplification was performed as

follows: pre-denaturation at 95 °C for 3 min, then 20 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min, and finally an additional elongation at 72 °C for 5 min. The PCR products were confirmed by agarose gel electrophoresis by using 3% agarose.

In order to obtain ssDNA library for next round of selection, streptavidin modified magnetic beads were used to separate biotinylated oligonucleotide strands from their complementary strands. 50 μg streptavidin modified magnetic beads were washed with binding and washing (B&W) buffer (10 mM Tris–HCl containing 1 mM EDTA, 2 M NaCl, pH7.5) for three times and suspended in 80 μL B&W buffer. Thereafter, 20 μL PCR products were added to the beads suspension, and incubated at room temperature for 15 min, then washed with B&W buffer. After further incubation in 100 mM NaOH at 37 °C for 15 min, the biotinylated strands kept adsorbed on streptavidin modified magnetic beads, while the complementary non-biotinylated ssDNA was eluted from the beads.

After the first round of selection, the obtained ssDNA was used as starting DNA pool in the next selection round. By using ethanolamine-coated epoxy beads, counter selection steps were performed to remove non-specifically adsorbed ssDNA every two selection rounds.

2.4. Binding assays of each round

After each round of selection, the obtained aptamers were amplified by using a biotinylated forward primer and a reverse primer. 0.4 μg PCR product in 100 μL binding buffer was incubated at 90 °C for 10 min, and then quickly mixed with streptomycin-coated magnetic beads. The mixture was incubated at 37 °C for 30 min. The beads were washed with binding buffer for five times, and the unbound DNA was removed. Then 20 μL HRP-conjugated streptavidin (1:2000) was added to the beads. After incubation at 37 °C for 20 min, the beads were washed for five times to completely remove unbound HRP-conjugated streptavidin. 100 μL of TMB solution was added to the beads, and allowed to react at 37 °C for 15 min. 2 M H_2SO_4 was used to terminate the reaction and the absorbance at 450 nm was then determined by using a microplate reader (Thermo Labsystems).

2.5. Cloning, sequencing and structural analysis

After eight rounds of selection, the recovered ssDNA was amplified with non-biotinylated primers. Then the PCR product was cloned, and 16 positive clones were picked and sequenced (Sangon Biotech Co. Ltd., Shanghai, China). Mfold software was used to analyze the secondary structures of the selected aptamers.

2.6. Determination of the dissociation constants (K_d)

Binding assays of the sequenced aptamers were performed by equilibrium filtration method as described previously [43]. Briefly, 10 μM streptomycin and a series of aptamers ranging from 0 to 1.5 μM were mixed in 200 μL binding buffer and incubated at 25 °C for 30 min. Then the solution was loaded into ultrafiltration centrifugal tube with the cutting molecular weight of 3000 Da, and centrifuged for 5 min at 12,000 rpm, allowing 100 μL of the solution to flow through the membrane. The filtrate through the membrane contained only free streptomycin, which was measured by HPLC (Agilent 1200) using Diamonsil C18 ($4.6 \times 250\ mm^2$) column. The mobile phase was 10 mM phosphate buffer containing 14 g/L sodium sulfate, 1.5 g/L sodium 1-octanesulfonate and 140 mL/L acetonitrile (pH 3.0). The absorbance at 205 nm of the eluate was detected and used to quantify streptomycin.

The dissociation constant was calculated by non-linear regression analysis using Eq. (1) [44,45] and 1 StOpt software:

$$y = B_{max} \cdot [\text{free ssDNA}]/(K_d + [\text{free ssDNA}]) \quad (1)$$

where y represents degree of saturation, B_{max} represents the maximum number of binding sites, [free ssDNA] represents the concentration of unbound ssDNA.

2.7. Evaluation of specificity of the aptamers

To investigate the specificity of the selected aptamers, neomycin, kanamycin, gentamicin and tetracycline modified epoxy magnetic beads were used, and the binding rates of the aptamers were measured. The antibiotics modified beads were firstly washed with binding buffer for five times, and suspended in 200 μL buffer. 2 μg aptamer in 200 μL binding buffer was incubated at 90 °C for 10 min and quickly added to the beads suspension. The mixture was incubated at room temperature for 30 min. The unbound ssDNA was washed with binding buffer for three times and collected together. The bound ssDNA was eluted with 150 μL of elution buffer by incubation at 80 °C for 10 min. The elution was repeated three times and the eluent was collected. The collected ssDNA (unbound and bound) was purified and quantified, and the binding rate of the aptamer can be determined.

2.8. Detection of streptomycin using AuNPs-based colorimetric assay

AuNPs (~13 nm diameter) were prepared as described previously [31]. 50 μL AuNPs (17.5 nM) was mixed with 100 μL aptamer STR1 (100 nM), and the solution was allowed to incubate for 1 h at room temperature. Then 100 μL of different concentrations of streptomycin was added to the above solution, respectively, followed by 30 min incubation at room temperature. Finally, 50 μL of 20 mM NaCl was added and UV–visible spectroscopy was performed to analyze streptomycin quantitatively.

To further confirm the specificity of the assay, 100 μL of 600 nM kanamycin, tetracycline, ampicillin and double distilled water, instead of streptomycin, were added to the above AuNPs-aptamer mixture and incubated under the same conditions. Then 50 μL of 20 mM NaCl was added and the UV–visible spectra were recorded.

2.9. Detection of streptomycin in honey samples

To detect streptomycin in honey samples, artificially contaminated honey was prepared by spiking standard streptomycin solution into diluted streptomycin-free honey. Different concentrations of calibrants were prepared by serial dilution with streptomycin-free honey. And different types of honey, including acacia honey, rape honey and vetch honey were used to prepare samples. Then AuNPs-based colorimetric assay was carried out as described above.

3. Results and discussion

3.1. In vitro selection

Streptomycin-coated magnetic beads were used to select ssDNA aptamers specific to streptomycin from a 79-mer initial ssDNA library including 35 random nucleotides. During the modification of streptomycin, the amount of streptomycin was measured by malt phenol colorimetric method. The bound streptomycin was estimated to be 0.6 μmol/mg beads according to the calibration curve and the absorbance change of streptomycin

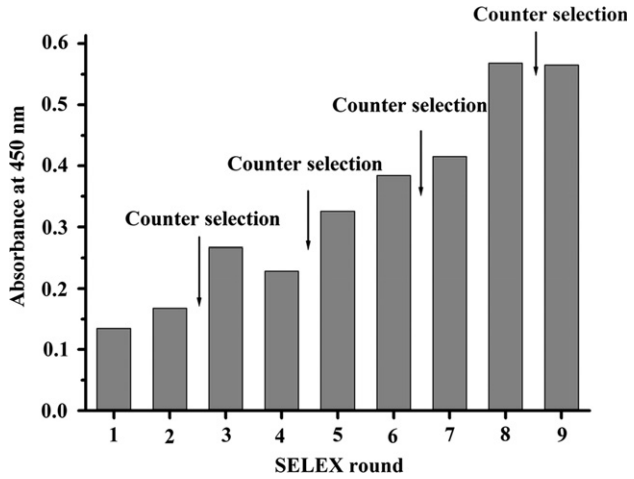

Fig. 1. Binding assays of the obtained aptamers after each selection round by using HRP mediated chromogenic reaction. The arrows indicate the counter selection rounds carried out by using ethanolamine-coated epoxy magnetic beads.

Table 1
35-nucleotides variable regions of the sequenced ssDNA aptamers. The conserved motifs are highlighted.

Aptamers	Random sequence of 35-nucleotides
STR1	**GGGG**TCT**GGTGTT**CTGCTTT**GTT**CTGTC**GGGT**CGT
STR3	TGAA**GGGT**CGACTCTAGAGGCA**GGTGTT**CCTCAGG
STR6	AGCTT**GGGTGGGG**CCACGTAGA**GGT**ATAGCTT**GTT**
STR12	TGTGT**GTTCGGT**GCTGTC**GGGT**T**GTT**TCTTC**GTTT**
STR13	T**GGGT**TGTCTAG**GTTGGT**ATGCTCAGTGCTAAATA
STR9	GCGAACTCGC**GTTTT**GTCTTTTTGCTTTG**GGTGTT**
STR2	GTGT**GTTT**GT**GGT**GTG**GTTTGTTT**GTGT**GTT**GTTT
STR14	ACAGCCTCTCGTGGATGCA**GTTTT**CGCGT**GGCGC**C
STR15	A**CGCC**CGCAGTCC**GTTTT**ATTTGTCTCATTGCTAC
STR4	TGCAGAGGATTACTATCATT**GGCC**ACGCTA**GTT**CC
STR8	**GGCC**TGTCACCGATCGCTAATTCATACTCTC**GTT**C
STR10	GCAAAGCTGATAATC**GTT**CTTT**GGCC**TGATATGTG
STR16	ATCCGTGA**GCCG**AGT**GGT**ACTTATAGCTCTCACAT
STR5	A**GGCC**CTCCAGTGGAGATTAAAC**GGT**TCGTCATCT
STR7	AGGGAGTCCATGTCGTAGTGAT**CGGC**TGTAAT**TGG**
STR11	C**CGCGG**AAGT**GGCGC**GCAAAGAG**GGT**TGGCATTCG

solution before and after the immobilization (Supplementary Fig. S1 and Table S1).

The selection was carried out for nine rounds. By using ethanolamine-coated epoxy magnetic beads, counter selection was performed after every two rounds of selection to remove aptamers specific to other groups on the beads. After each round, binding assay was performed to evaluate the relative affinity of the selected aptamers for the target by using biotinylated aptamers and streptavidin-HRP amplified assay system. As streptomycin-specific aptamers were enriched during selection rounds, the amount of biotinylated aptamers adsorbed on streptomycin-coated beads increased, which further increased the amount of adsorbed HRP-conjugated streptavidin. The process can be characterized by HRP catalyzed chromogenic reaction. Fig. 1 exhibits the corresponding absorbance at 450 nm after each selection round. The result shows that the absorbance increased from 0.135 to 0.568 during eight rounds of selection and no longer increased in further selection round, which may be attributed to the saturation of the binding sites on streptomycin-coated beads. Therefore, aptamers pool from the eighth round were amplified by PCR and then cloned.

3.2. Characterization of the selected aptamers

Sixteen random positive clones were identified and sequenced. The 35-mer variable regions of these aptamers are listed in Table 1. From sequence analysis, aptamers STR1, STR3, STR6, STR12 and STR13 were found to have high sequence homology. The variable regions of these aptamers possess a G-rich conserved sequence motif "GGGT". The secondary structures of the aptamers were predicted by using Mfold software (Fig. 2). In mimic diagrams, green regions represent fixed sequences and blue regions represent random sequences. In these secondary structure patterns, at least two loops together with stems exist as basic motifs. Meanwhile, the conserved "GGGT" usually locates at loop section, which appears to be significant for binding to the target.

Binding assays of the aptamers were performed by equilibrium filtration method. Fixed concentration of streptomycin was mixed with varying concentrations of aptamers. After ultrafiltration centrifugation, free streptomycin in the filtrate was measured by HPLC. The chromatograms of streptomycin solution with and without the addition of aptamer were shown in Fig. S2. By non-linear regression analysis, the K_d values of STR1, STR3, STR6 and STR12 were determined, which are of 199.1 nM, 221.3 nM, 272.0 nM and 340.64 nM, respectively. The non-linear regression curve of STR1 is shown in Fig. 3. Among the sequenced aptamers, STR1 has the highest affinity for the target.

Then the specificity of STR1 was investigated by using streptomycin, kanamycin, neomycin, gentamicin or tetracycline modified epoxy magnetic beads. As shown in Fig. 4, the binding rate of STR1 with streptomycin is 75.6%, while the value is less than 10% with other aminoglycoside antibiotics such as kanamycin, neomycin and gentamicin. The binding rate of STR1 with other families of antibiotics, such as tetracycline can be neglected. Therefore, STR1 is highly specific to streptomycin.

3.3. Detection of streptomycin using AuNPs-based colorimetric assay

As is well reported [24,46], ssDNA can be adsorbed onto the surface of AuNPs through electrostatic interaction. The ssDNA-coated AuNPs exhibit excellent stability in high concentration of NaCl solution, while bare AuNPs aggregate under the same condition. In this work, STR1 serves as stabilizer of AuNPs, preventing the aggregation of AuNPs in NaCl solution. However, when streptomycin exists, it combines with STR1 specifically, which removes the ssDNA layer from AuNPs competitively. Then the color of the solution turns from red to purple due to the AuNPs aggregation. The aggregation of AuNPs triggered by streptomycin was confirmed by TEM analysis (Supplementary Fig. S3). The procedure was further characterized by UV–visible absorption spectroscopy, and the concentration of streptomycin was quantified by the absorbance at 520 nm.

As shown in Fig. 5A, in the control experiment, in the absence of streptomycin, STR1 stabilized AuNPs in NaCl solution exhibit a typical SPR absorption peak at 520 nm in the UV–visible spectrum, while the color of AuNPs solution appears in red. As different concentrations of streptomycin were added, the color of AuNPs solution turned from red to purple gradually. The color change was further confirmed by UV–visible spectra. The absorbance of AuNPs apparently decreases with the increased concentration of streptomycin, owing to the aggregation of STR1-stripped AuNPs in NaCl solution. Meanwhile, the maximum absorption wavelength of AuNPs undergoes a slight red-shift, which is in accordance with the aggregation. Furthermore, the relationship between the absorbance at 520 nm in UV–visible spectra and the concentration of streptomycin was studied, and a linear relationship was found in streptomycin concentration range of 0.2–1.2 μM (Fig. 5B). The linear regression equation is $y=-7.629e-5x+0.8254$, $R^2=0.9912$, where y represents the absorbance at 520 nm in the UV–visible spectra, x represents the concentration of streptomycin (nM). The absorbance maintains relatively constant as the concentration of streptomycin is

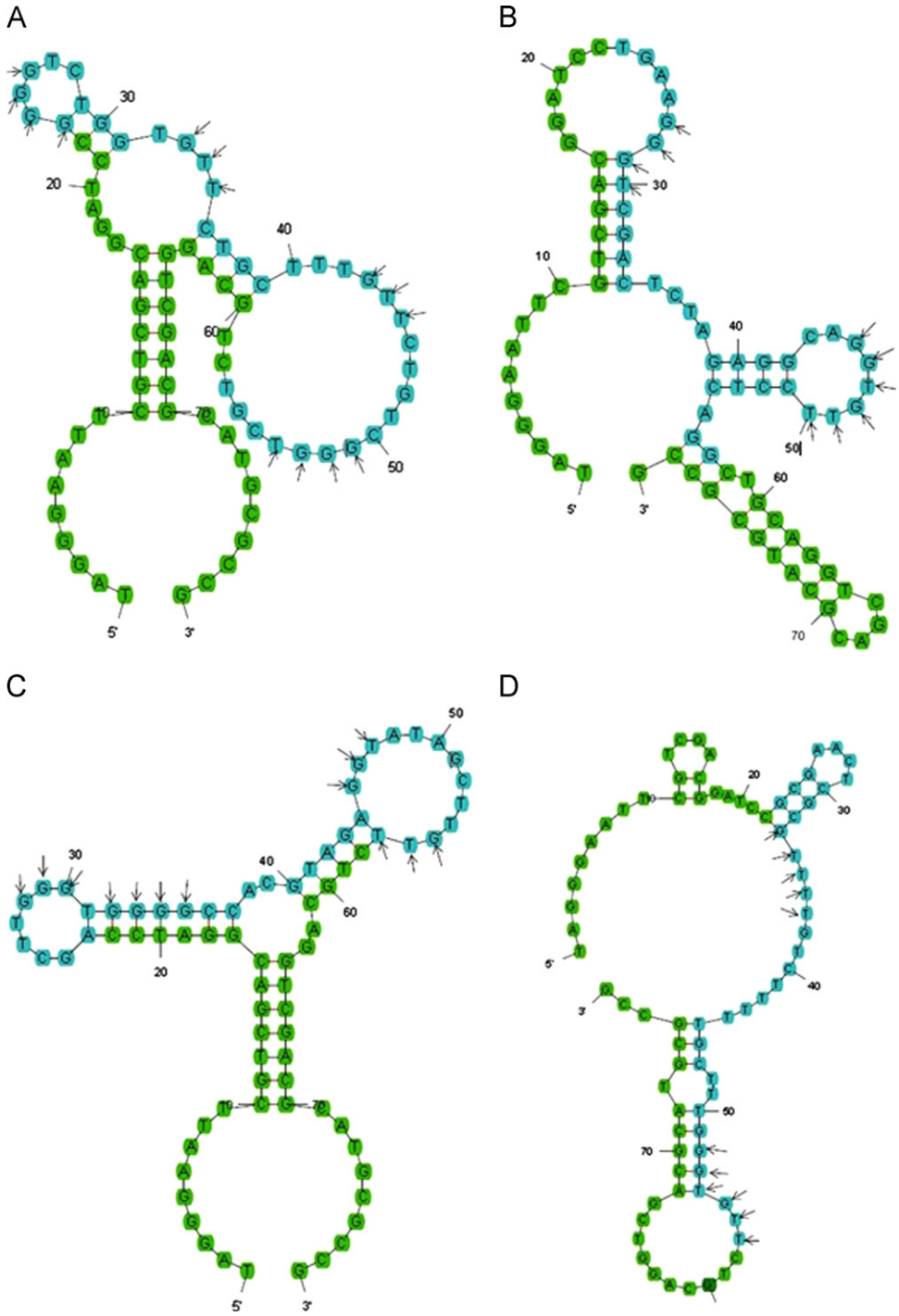

Fig. 2. Secondary structure models of streptomycin aptamers STR1 (A), STR3 (B), STR6 (C), and STR12 (D) predicted by Mfold software. Green regions represent fixed sequences and blue regions represent random sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

higher than 1.4 μM. Therefore, AuNPs-based colorimetric assay can be used to detect streptomycin with high sensitivity.

To verify the assay with widely recognized methods, different concentrations of streptomycin solution were prepared and analyzed by AuNPs-based colorimetric assay and HPLC, respectively. The results are shown in Table 2. From the results we can conclude that both methods show excellent accuracy. Though the RSD of AuNPs-based colorimetric assay (1.48–1.82%) is a little higher than that of HPLC (0.75–0.93%), it is still good enough for general applications. Meanwhile, the process of AuNPs-based colorimetric assay is much easier than that of HPLC, and the detection limit of the assay is much lower than that of HPLC.

In order to estimate the specificity of the assay, double distilled water, 600 nM of streptomycin, kanamycin, tetracycline, and ampicillin were added to STR1 stabilized AuNPs, respectively. As shown in Fig. 6, an obvious color change of AuNPs can be observed in the presence of streptomycin. However, the color of AuNPs remains unchanged in the presence of either double distilled water or other antibiotics. The results indicate that the assay is highly specific to streptomycin.

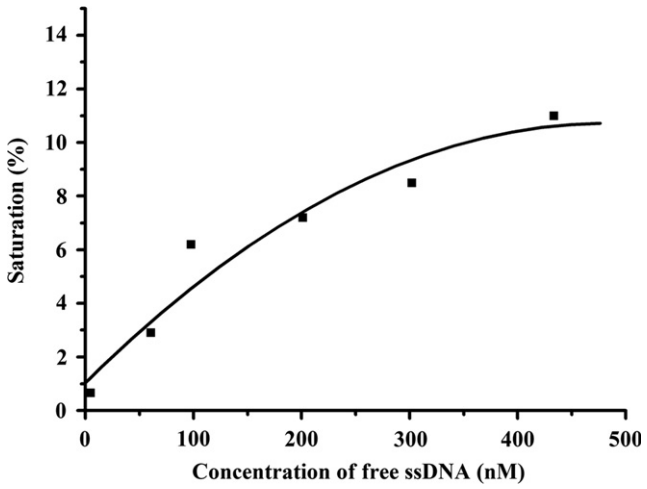

Fig. 3. Non-linear regression analysis of the K_d value of STR1. The saturation curve was obtained by plotting the saturation of streptomycin against the concentration of free ssDNA.

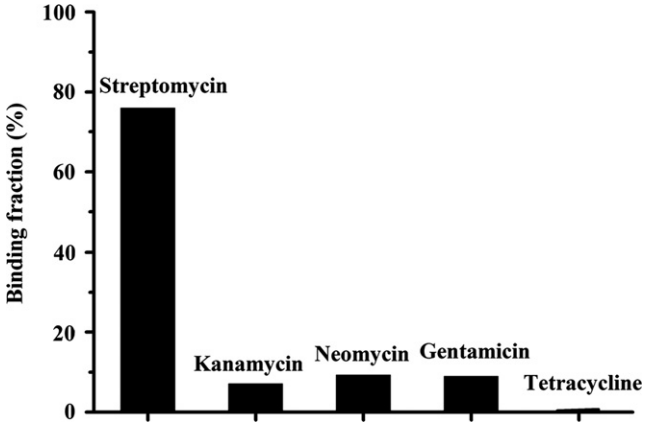

Fig. 4. Binding specificity of aptamer STR1. The binding fractions of STR1 with streptomycin, kanamycin, neomycin, gentamicin and tetracycline were determined.

3.4. Detection of streptomycin in honey

To achieve the aim of detection of streptomycin in real food samples, the assay was migrated to detect streptomycin in artificially contaminated honey, which was prepared by spiking standard streptomycin solution into diluted streptomycin-free honey. Generally, the color and viscosity of the food matrices can disturb the AuNPs-based colorimetric assay. For the complicated samples such as milk, pretreatments are necessary for AuNPs-based assay. For quasi-transparent and homogeneous samples such as honey, high viscosity can be the key factor effecting on the assay. In our experiments, when undiluted honey was mixed with aptamer stabilized AuNPs, AuNPs were suspended in the high-viscous mixture and the aggregation cannot occur. However, as diluted honey was used to prepare artificially contaminated honey, the interferences from the matrix can be eliminated. Artificially streptomycin-contaminated honey prepared by both five-times (Supplementary Fig. S4) and ten-times diluted acacia honey (Fig. 7) were successfully analyzed.

The streptomycin-contaminated acacia honey was added to STR1 stabilized AuNPs and incubated under the same conditions with the standard streptomycin samples. Then the UV–visible spectra were recorded after the addition of NaCl. As shown in Fig. 7A, the UV–visible spectrum of AuNPs exhibits no observable change in the presence of diluted honey. However, the absorbance

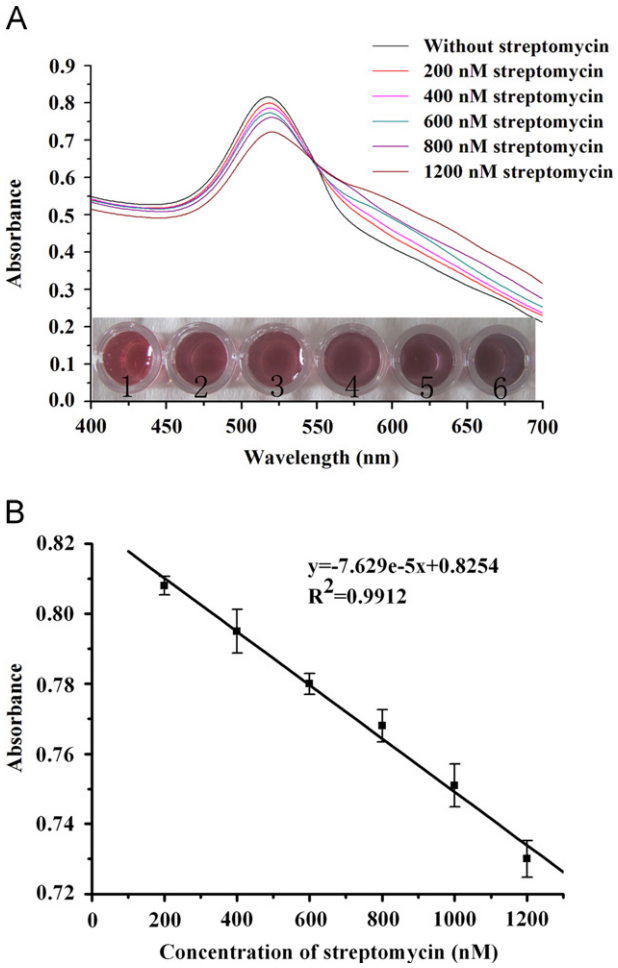

Fig. 5. (A) UV–visible spectra of AuNPs in NaCl solution in the presence of different concentrations of streptomycin. Inset shows the corresponding color changes of AuNPs in the presence of different concentrations of streptomycin (lane 1: 0 nM, lane 2: 200 nM, lane 3: 400 nM, lane 4: 600 nM, lane 5: 800 nM, lane 6: 1200 nM). (B) The derived linear relationship between the absorbance at 520 nm in the UV–visible spectra and the concentration of streptomycin.

Table 2
The comparison of AuNPs-based colorimetric assay and HPLC assay. Mean value and RSD were from five independent experiments.

Streptomycin added into the solution (μM)	10	20	30
Mean detection value of AuNPs-based assay (μM)	10.04	19.74	30.20
RSD of AuNPs-based assay (%)	1.48	1.73	1.82
Mean detection value of HPLC assay (μM)	9.98	19.84	30.12
RSD of HPLC assay (%)	0.75	0.93	0.86

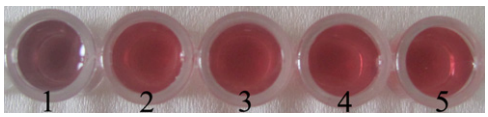

Fig. 6. Color of AuNPs in the presence of double distilled water or 600 nM of different antibiotics (lane 1: streptomycin, lane 2: kanamycin, lane 3: double distilled water, lane 4: tetracycline, and lane 5: ampicillin).

of AuNPs at 520 nm apparently decreases with the increased concentration of streptomycin in honey samples, which is just the same with those standard streptomycin samples. And the color change of AuNPs can also be observed distinctly. Furthermore, in the concentration range of 0.2–1.2 μM, a linear relationship was also found between the absorbance at 520 nm and the concentration of streptomycin in honey (Fig. 7B). The linear regression

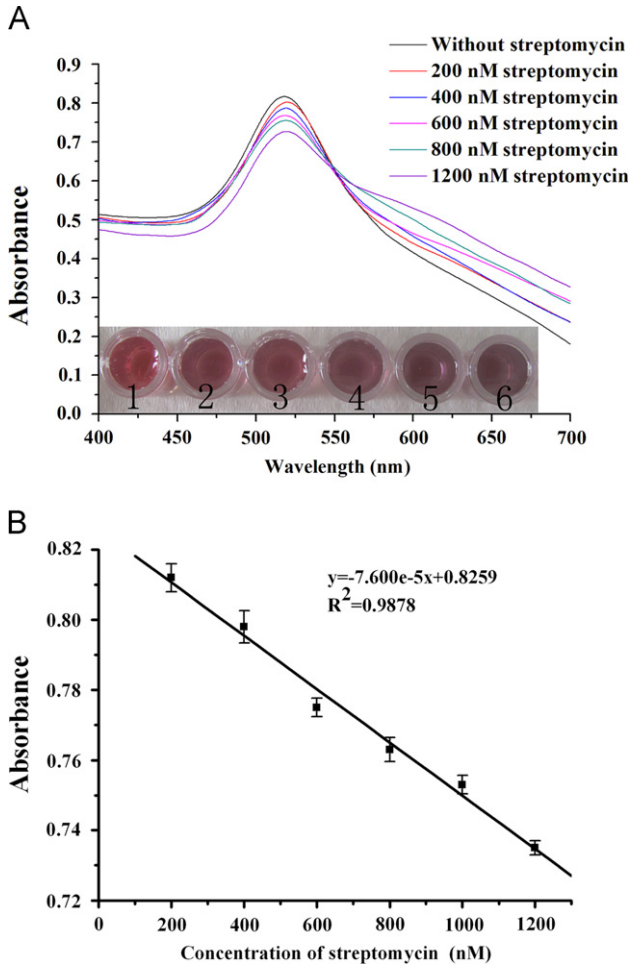

Fig. 7. (A) UV–visible spectra of AuNPs in NaCl solution in the presence of honey samples with different concentrations of streptomycin. Inset shows the corresponding color changes of AuNPs in the presence of different concentrations of streptomycin-contained honey samples (lane 1: 0 nM, lane 2: 200 nM, lane 3: 400 nM, lane 4: 600 nM, lane 5: 800 nM, and lane 6: 1200 nM). (B) The derived linear relationship between the absorbance at 520 nm in the UV–visible spectra and the concentration of streptomycin in honey.

equation is $y=-7.600e-5x+0.8259$, $R^2=0.9878$, where y and x have the same meanings as above. It is found that the slope and the intercept of the linear regression equations of standard streptomycin samples and honey samples are almost equivalent, which suggests that the honey matrix does not interfere the assay of target streptomycin.

The assay was further confirmed by using different types of contaminated honey as samples. Besides acacia honey, artificially streptomycin-contaminated rape honey and vetch honey have also been analyzed under the same conditions. And the similar UV–visible spectra and linear relationships were obtained (Supplementary Figs. S5 and S6). Therefore, the screened aptamer and the established method are suitable for the applications of streptomycin detection in food samples.

4. Conclusion

ssDNA aptamers that bind to streptomycin with high affinity and high specificity were screened and identified from a random oligonucleotides library by using affinity magnetic beads-based SELEX. A total of eight rounds of SELEX were performed, after which 16 ssDNA with different sequences were identified. After the determination of K_d value and the estimation of specificity, STR1 was chosen as the ideal aptamer for analytical applications. To our knowledge, it is the first reported ssDNA aptamer specific to streptomycin. Based on AuNPs-based colorimetric assay, streptomycin in standard solution as well as in honey samples can be detected in the range of 0.2–1.2 μM. With excellent specificity and sensitivity, the screened aptamer and the established method exhibit great promise in the applications of antibiotics residue assay in food products.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.01.064.

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