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A direct competitive assay-based aptasensor for sensitive determination of tetracycline residue in Honey

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ABSTRACT

Tetracycline (TC) is a common antibacterial agent used for prevention and control of animal diseases. The increasing concern about TC residue in food demands high-performing analytical techniques for food quality assessment. Biosensors represent a promising tool for food safety analysis as they can fulfill some demand that the conventional methods do not attain. In this study, a novel colorimetric aptasensor was developed for sensitive detection of TC in honey. The aptasensor was based on a modified direct competitive enzyme-linked aptamer assay (*dc*-ELAA) scheme utilizing a 76mer single-stranded DNA (ssDNA) aptamer selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The optimized aptasensor showed a good limit of detection (LOD of 0.0978 ng/mL), a wide linear range (0.1–1000 ng/mL) toward TC in honey, with good recoveries (92.09–109.7%) in TC-spiked honey, and was compared with an indirect competitive assay-based aptasensor and validated with a standard ELISA. The biosensor based on *dc*-ELAA with good limit of detection and simplicity can be applied for high-throughput detection of TC in food.

1. Introduction

Biosensors, which have experienced dramatic evolution since 1962 with the first introducing of enzyme electrodes [1], are a sub group of chemical sensors composed of a biological recognition element coupled to a chemical or physical transducer. Biosensors can be grouped into categories in agreement to the type of biological component or the mode of signal transduction or both of them, such as microbial biosensors, affinity-based biosensors, electrochemical biosensors, optical biosensors, etc [2]. Different biosensors have been developed for clinic, environment, agriculture and biotechnology in the past few years [3–9], and these biosensors demonstrate prominent advantages as alternatives to traditional methods in terms of specificity, simplicity, sensitivity, relative low cost, and potential for portable equipment construction [10].

Aptamers, first reported in 1990 [11,12], are artificial short single-stranded oligonucleotides of DNA or RNA selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Aptamers appear to be superior alternatives to antibodies or other biological recognition elements with the advantages of high affinity, high specificity, stable selecting process with uniform reactivity and not involved with immunogenicity [13–16], adaptive folding and structural modulation upon the target molecules [17,18], and easy modification [19].

Aptasensors, a kind of affinity-based biosensors with aptamer as recognition element and commonly designed with different signal transducers, such as colorimetric [20,21], optical [22–24], and electrochemical [25,26], have emerged as a powerful tool in the domains of diagnostics and therapeutics, environmental monitoring, and food safety analysis.

Tetracycline (TC), which is a member of the most common broad-spectrum tetracycline group of antibiotics (tetracyclines, TCs), is extensively used as veterinary drug and feed additive, thus tends to accumulate in finished food products, posing serious risk to customers' health. During the production of honey, TC is often used for the treatment of bacterial brood diseases in apiculture, such as American foulbrood (*Bacillus larvae*) and European foulbrood (*Streptococcus pluton*) [27]. To ensure the security of consumers, some countries have set maximum residue limit (MRL) for TC in honey, and others ban use of TCs with bees at any level. In China, the MRL for TC in honey was formerly set [28] and updated by new regulation [29] at 0.05 mg/kg. Traditional chromatographic w/wo mass spectrometric methods have been reported for the quantification and verification of TC with precise results [30–32]. On the other hand, antibody-based methods for rapid screening of TC in food samples have also been developed

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with simplicity and high-throughput capability, such as enzyme-linked immunosorbent assay (ELISA) or gold immuno-chromatographic assay (GICA). However, the former chromatographic methods are expensive, require tedious sample-preparation and extended analysis time, and the latter screening techniques have some drawbacks in terms of antibody production, preservation, ethical problems with the use of animals, etc [33]. Thus there is an imperative need to develop efficient determination methods for detection of TC in honey.

In this study, we developed an optimized aptasensor as a sensitive and selective biosensor for the determination of TC in honey based on a simple direct competitive enzyme-linked aptamer assay (*dc*-ELAA) scheme. We improved the protocol with direct competitive format and successfully obtained an excellent limit of detection data. The present aptasensor involving no complicated sample extraction steps is more suitable for practical screening of TC residue in honey with a broad linear range. Additionally, confirmatory comparison of *ic*-ELAA (indirect competitive ELAA) and *dc*-ELAA with standard ELISA test for tetracycline detection in honey was made for the first time to demonstrate the merits of the two competitive assay formats and to provide two aptasensors for TC detection in honey matrix.

2. Materials and methods

2.1. Reagents

Streptavidin (SA), Bovine serum albumin (BSA) and 3, 3′, 5, 5′-tetramethylbenzidine dihydrochloride (TMB) were purchased from KPL (Gaithersburg, MD, USA). Tetracycline standard (TC) and Hammerstein bovine casein (Casein) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). The aptamer-3′-biotin (Mw. (23747.43), mp (83.77 °C)) was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the 76-oligomer sequence is (Fig. 1): 5′-CGTACGGAATTCGCTAGCCCCCCGGCAGGCCACGGCTTGGGTTGGTCCCACTGCGCGTGGATCCGAGCTCCACGTG-3′. Two kinds of horseradish peroxidase labeled tetracycline (TC–HRP and TC–BSA–HRP) and a tetracycline ELISA kit were purchased from Shandong LvDu Bio-sciences & Technology Co., Ltd (Shandong, China). All chemicals were of analytical grade and buffers were prepared with ultrapure water and filtered using 0.22 μm membrane filter before use.

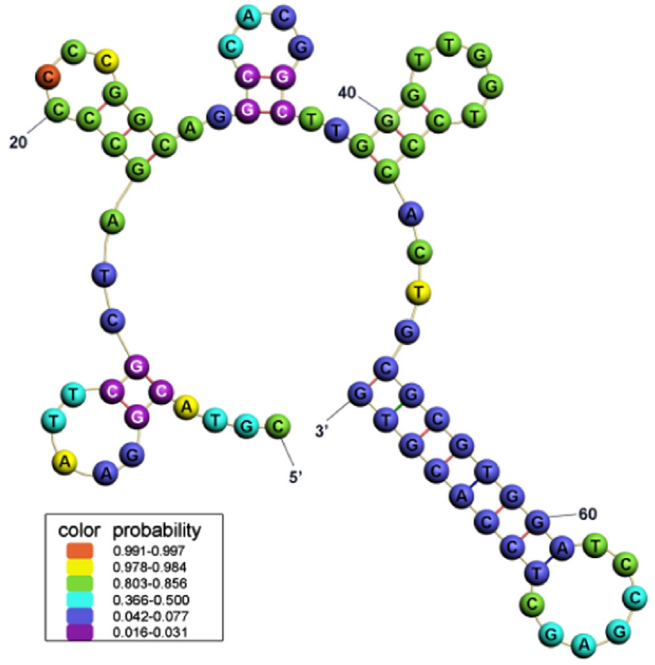

Fig. 1. Secondary structure of the 76mer ssDNA aptamer predicted by m-fold according to free energy minimization algorithm [36]. Color annotation means the probability.

2.2. Buffers

Coating buffer: bicarbonate buffer (0.05 M CB, pH 9.6). Dilution buffer for blocking agent: phosphate buffer saline (10 mM PBS, pH 7.4). Washing buffer: Tris-T buffer (10 mM Tris, 0.05% Tween 20, pH 7.6). Binding buffer for aptamer and TC (Binding Buffer,100 mM NaCl, 20 mM Tris–HCl (pH 7.6), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM KCl, 0.02% Tween-20 [34]); McIlvaine-Na_2EDTA buffer (0.1 M M-E, 12.93 g of citric acid, 37.33 g of Na_2EDTA, 22.38 g $Na_2HPO_4 \cdot 12\ H_2O$ in 1 L of ultrapure water, pH 4.0).

2.3. Instrumentation

ELAA was performed in 8 well Flat-Bottom Immuno Plates (Nunc, Denmark). The plates were incubated in a *SPX-70* biochemical incubator (National Science Instrument and Technology Co., Ltd., Beijing, China), shaking with a *MX-M* 96-well plate mixer (Dragon Laboratory Instruments, Ltd., Beijing, China). Absorbance was measured using *ELx800* absorbance microplate reader (BioTek Instruments, Inc., Winooki, Vermount, USA) at both 450 nm and 630 nm. The plates were washed using *ELx50* microplate strip washer (BioTek Instruments, Inc., Winooki, Vermount, USA). The protein content of honey was measured by Nanodrop 2000C (Thermo Fisher Scientific Nanodrop 2000C, Wilmington, Delaware USA) at 280 nm. The ultrapure water was prepared by Thermo Scientific Barnstead GenPure Water Purification System (Thermo Electron LED GmbH, Stockland 3, D-56412 Niederelbert). Centrifugation was performed in Sigma refrigerated centrifuge 3K15 (Sigma Laborzentrifugen GmbH, An der Unteren Söse 50, D-37520 Osterode, German).

2.4. Optimization of assay conditions before competition

At the beginning of the optimization, a checkerboard study was performed to evaluate the concentration of SA and blocking agents, two critical factors respectively influencing on the specific and nonspecific immobilization of biotinylated aptamer on microtiter plates. The satisfactory concentration of SA was 1 μg/mL, and the superior condition of 1% (w/v) Casein or 2% (w/v) BSA was selected as blocking agent (data not shown). Based on this, concentrations of binder (aptamer) and competitor (TC–HRP or TC–BSA–HRP) were further analyzed. The labeled aptamer was prepared in concentrations of 1–50 nM and the TC–HRP conjugates was diluted to 1:800, 1:1600, 1:3200 and 1:6400. In addition, the specific binding ability of aptamer with TC in this aptasensor system was analyzed w/wo thermal treatment. The thermal treatment means heating the aptamer at 95 °C for 10 min and then cooling rapidly in ice for 10 min before the immobilization of the biotinylated ssDNA.

2.5. Direct competitive enzyme-linked aptamer assay (dc-ELAA) for TC

The direct competitive assay was performed as shown in Fig. 2. SA was diluted to 1 μg/mL in CB coating buffer and 100 μL/well of SA was coated on the microtiter plates at 4 °C for 16 h. To remove unbound SA, wells were washed with 300 μL/well of Tris-T washing buffer for three times. The plates were then blocked with 200 μL/well of 1% Casein or 2% BSA in 10 mM PBS for 90 min at 37 °C with mild shaking, and the unbound protein was removed. Subsequently, 100 μL/well biotinylated aptamer in Binding Buffer

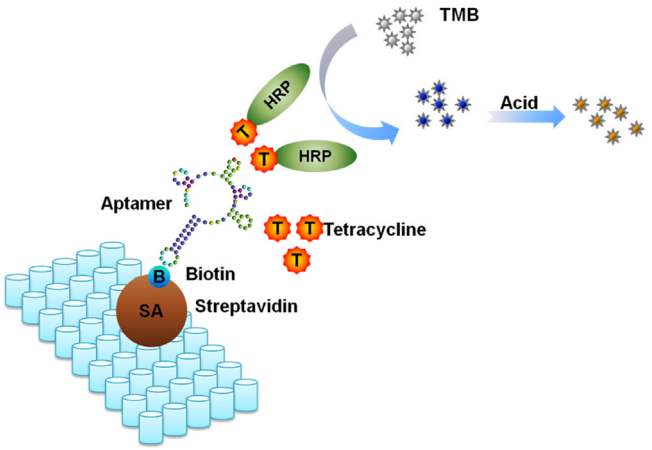

Fig. 2. Schematic illustration of *dc*-ELAA. Competition between TC–HRP and free TC in buffer or honey for the immobilized biotin labeled aptamer, and subsequent enzyme tracer detection.

and 50 μL/well various concentrations of TC solutions from 1×10^{-3} to 1×10^{4} ng/mL were added and incubated for 45 min with mild shaking at 25 °C. After washing, 100 μL/well HRP labeled TC diluted in Binding Buffer was added to the plates, and was allowed to bind with the immobilized aptamer for 30 min at 25 °C. After removing the TC–HRP, 100 μL/well TMB solution was added and incubated for 20 min at 37 °C. The color of the solution changed from colorless to blue and turned to yellow when adding 50 μL of 2 M H_2SO_4 to stop the color reaction. The absorbance was measured immediately at 450 nm with 630 nm as the reference wavelength. Each step was protected from light with silver paper. The corresponding test inhibition values were computed according to section 2.7.

2.6. Pretreatment for the analysis in Honey samples

Honey samples were purchased from local supermarket and stored at RT before use. The metal ions such as calcium, iron, magnesium, zinc [35] and protein (observed by Nanodrop 2000C) in honey will have negative influence on the combination of TC and aptamer. For removal of matrix effect, honey samples (5 g) were mixed with 20 mL of 0.1 M M-E buffer and 2 mL 0.5% trichloroacetic acid (TCA) for 5 min using a vortex mixer, then homogenized by ultrasonic for 5 min, and centrifugated at 4 °C for 20 min at 4000 rpm. Then 4 M NaOH was added dropwise to the supernatant to adjust the pH to 7.6. Finally, samples were stored at 4 °C in the dark and diluted with Binding Buffer (sample: buffer=1:5) before use. The detection of TC in honey was validated by *dc*-ELAA as described in section 2.5. TC standard was dissolved and diluted into concentrations from 1×10^{-3} to 1×10^{4} ng/mL using honey solutions.

2.7. Data processing

2.7.1. Significance analysis

Significance analysis between different conditions was characterized by *P* value using two-tailed T-tests and marked using different colors in Fig. 2.

2.7.2. Inhibition values A/A_0

The absorbance was measured immediately at 450 nm with 630 nm as the reference wavelength. The optical density of each well, denoted by *B*, was consistent with the result of absorbance at 450 nm minus absorbance at 630 nm. B_0 means the optical density value of the non-competitive control (with no TC). B_{blank} means the optical density value of the blank control (with no aptamer or TC). To reflect the actual competitive effect and improve the sensitivity, the absorbance of the competitive system is defined by *A*, namely *B* minus B_{blank}. The final absorbance values were converted into their corresponding test inhibition values A/A_0.

2.7.3. LOD

The LOD was defined as the concentration corresponding to 3 standard deviations below the mean absorbance from non-competitive control.

3. Results and discussions

3.1. Optimization of assay conditions

Prior to the competition assay, detailed assay conditions were investigated for the optimized method. The immobilized SA was exposed to various concentrations (1–50 nM) of biotinylated aptamer. The absorbance increased with the growing concentration of aptamer until 20 nM (Fig. 3(A)). As 1 nM aptamer led to a relatively lower absorbance, which was not considered as a better choice for a wider linear range, 2–20 nM aptamer was applied for the subsequent optimization. As shown in Fig. 3(B), the satisfactory dilution ratio of TC–HRP was 1:1600 to obtain an absorbance around 1.0 in order to get dose-curves with desired sensitivity [36]. Several studies reported that TC can form chelate compounds with BSA [37–39]. Thus some ELISA kits manufacturers try another novel kind of horseradish peroxidase labeled tetracycline (TC–BSA–HRP) to improve the sensitivity of the detection system and make more binding sites of TC exposed for antibody. However, TC–BSA–HRP did not perform well in the present biosensor employing aptamer as the recognition element (Fig. 3(C)), thus TC–HRP was selected as the enzyme tracer. The pretreatment of heating ssDNA at 95 °C for 10 min and then cooling rapidly in ice for 10 min was performed in order to denature and block the aptamer in its unfolded structure [40]. Single DNA or RNA tends to be more ordered and comes into adaptive folding from uncertain conformation to energy-efficient three-dimension structures when meeting with their targets in solution [17,41]. Moreover, the denaturation can also make the biotin label available for the interaction with streptavidin. Thus comparison was made between the aptamer w/wo a thermal treatment before immobilization. Although the result showed no significant difference between the two kinds of usage mode (Fig. 3(D)), the absorbance of no treatment control was slightly smaller. To the authors' experience, it is better to apply the thermal treatment to aptamer before it binds with TC, especially after a prolonged preservation period.

3.2. Dose-response curves in buffer

The *dc*-ELAA in this study was not merely an analytical method with similar protocol to ELISA, changing the recognition element from antibody to aptamer. The detailed implementation scheme should be modified in light of the adaptive folding capability of aptamer. Thus comparison between two different operation schemes when adding aptamer and TC to wells was made as shown in Fig. 4(A). One dose-curve was obtained by adding 100 μL/well biotinylated aptamer and 50 μL/well various concentrations of TC solutions simultaneously and incubated for 45 min with mild shaking at 25 °C, then TC–HRP for 30 min. The other curve was gained by adding 100 μL/well biotinylated aptamer incubating with SA for 45 min, and then 50 μL/well various concentrations of TC for 30 min with mild shaking at 25 °C, then TC–HRP for 30 min. The former performed higher sensitivity

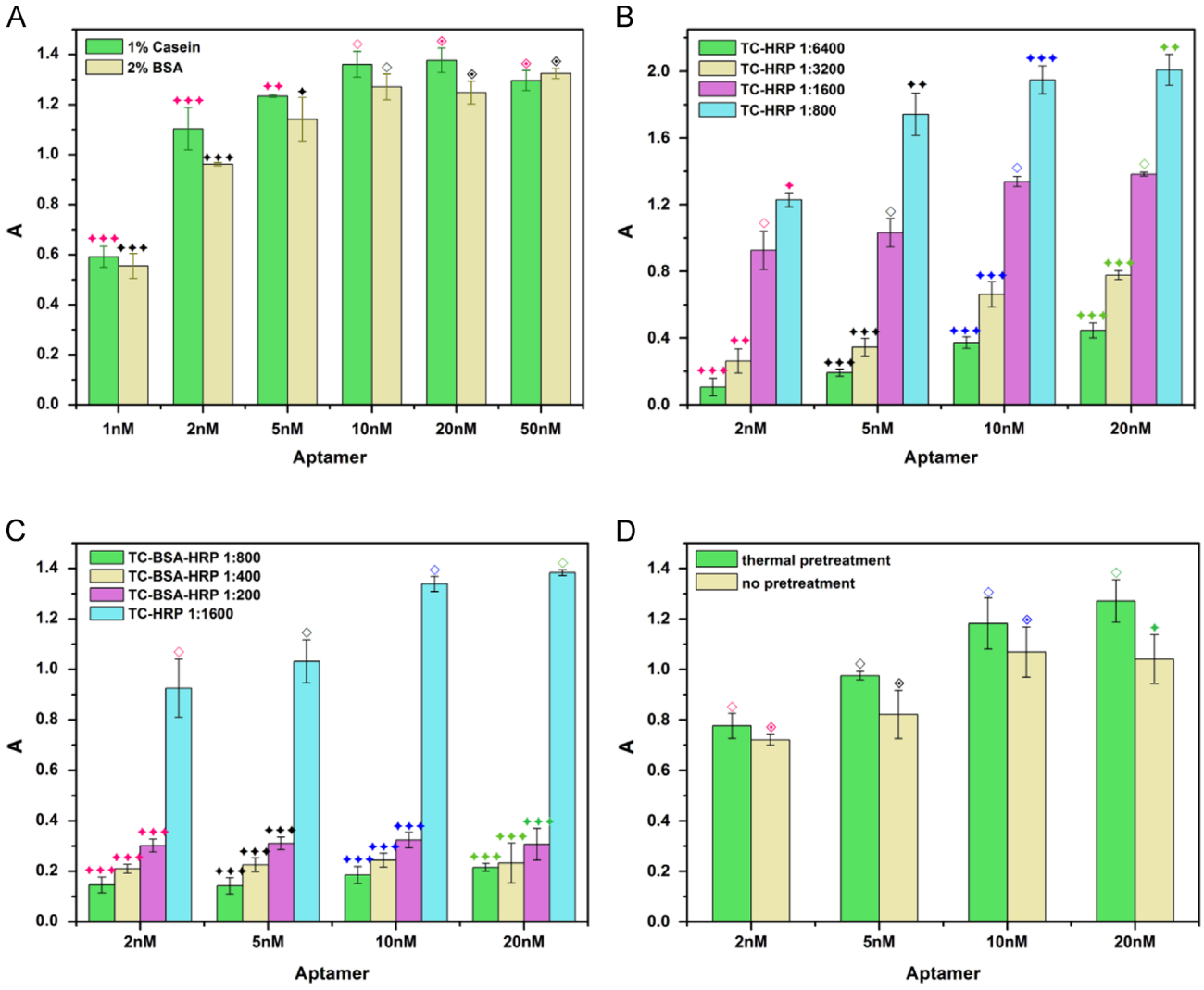

Fig. 3. Optimization of *dc*-ELAA assay conditions. Detailed working conditions were evaluated with 1μg/mL of SA. (A) Absorbance of 1–50 nM aptamer using 1% Casein or 2% BSA as blocking agent. (B) Absorbance of 2, 5, 10, 20 nM aptamer with different dilution ratio of TC–HRP. (C) Absorbance of 2, 5, 10, 20 nM aptamer under the condition of different enzyme tracers. (D) Absorbance of 2, 5, 10, 20 nM aptamer with thermal treatment or not. Significance analysis was characterized according section 2.7 and marked using different colors. The symbol ◇ denotes control group, ◈ denotes $P > 0.05$, ✦denotes $P < 0.05$, ✦✦denotes $P < 0.01$, ✦✦✦denotes $P < 0.001$.

owing to aptamer's advantage of adaptive folding. Aptamer can fold and bind upon the targets [17–18] and can be structurally modulated upon the target molecules [33]. In the present study, thermal-cooling treatment blocked the aptamer in unfolded state, and aptamer folded into specific secondary structure for TC with the presence of TC. Jeong and Rhee Paeng [40] developed an aptasensor for the determination of TC in bovine milk using the second scheme in Fig. 4(A), however, the LOD (15.7 ng/mL) was higher than the LOD (0.0978 ng/mL) in the present study and their dynamic range (88.8 ng/mL–44.4 μg/mL) was relatively narrower than that of this study (0.1–1000 ng/mL). Hence TC solutions had better be added with aptamer at the same step to induce an adaptive folding, and moreover the pre-incubation between TC and aptamer could contribute to obtain a more sensitive competitive curve.

The sensitivity of the competition assay depends on the immobilized aptamer concentration on the solid surface and the suitable concentration of competitor, TC–HRP conjugates. Concentrations of aptamer and TC–HRP were controlled, and dose-response curves were performed. Three concentrations of aptamer (2, 5, 10 nM) were used to investigate the sensitivity for TC detection. The 5 nM concentration of aptamer was found to provide optimum sensitivity and sufficient absorbance values for an easily detectable signal. The results in Fig. 4(B) demonstrated that sensitivity increased as lower concentrations of aptamer were utilized. However, 2 nM aptamer showed a relatively narrower linear range than 5 nM because the lower concentration of aptamer provided less binding sites for TC and is easier to be fully occupied, while the higher concentration of 10 nM aptamer gave rise to low sensitivity in competition. As shown in Fig. 4(C), the selected dose-curve was compared with other two condition-changed curves, one with 2% BSA as blocking agent and the other with 1:3200 of TC–HRP as competitor. The dose-curve utilizing 2% BSA provided similar working range with the selected dose-curve, but had a lower sensitivity. This can be explained by the combination of TC and BSA [37–39]. The other dose-curve was obtained using a lower concentration of TC–HRP to optimize the assay performance. However, the inhibition difference between the high and low doses of TC was too small to be valuable. In light of the sensitivity and working linear range of the dose-curve, 5 nM aptamer, 1:1600 TC–HRP and 1% Casein were chosen as suitable conditions in the aptasensor and to be used for TC detection in honey samples.

3.3. Detection of TC in Honey using direct competitive ELAA

Matrix effects may influence the aptamer-analyte interaction and reduce the detection sensitivity. McIlvaine-Na_2EDTA buffer

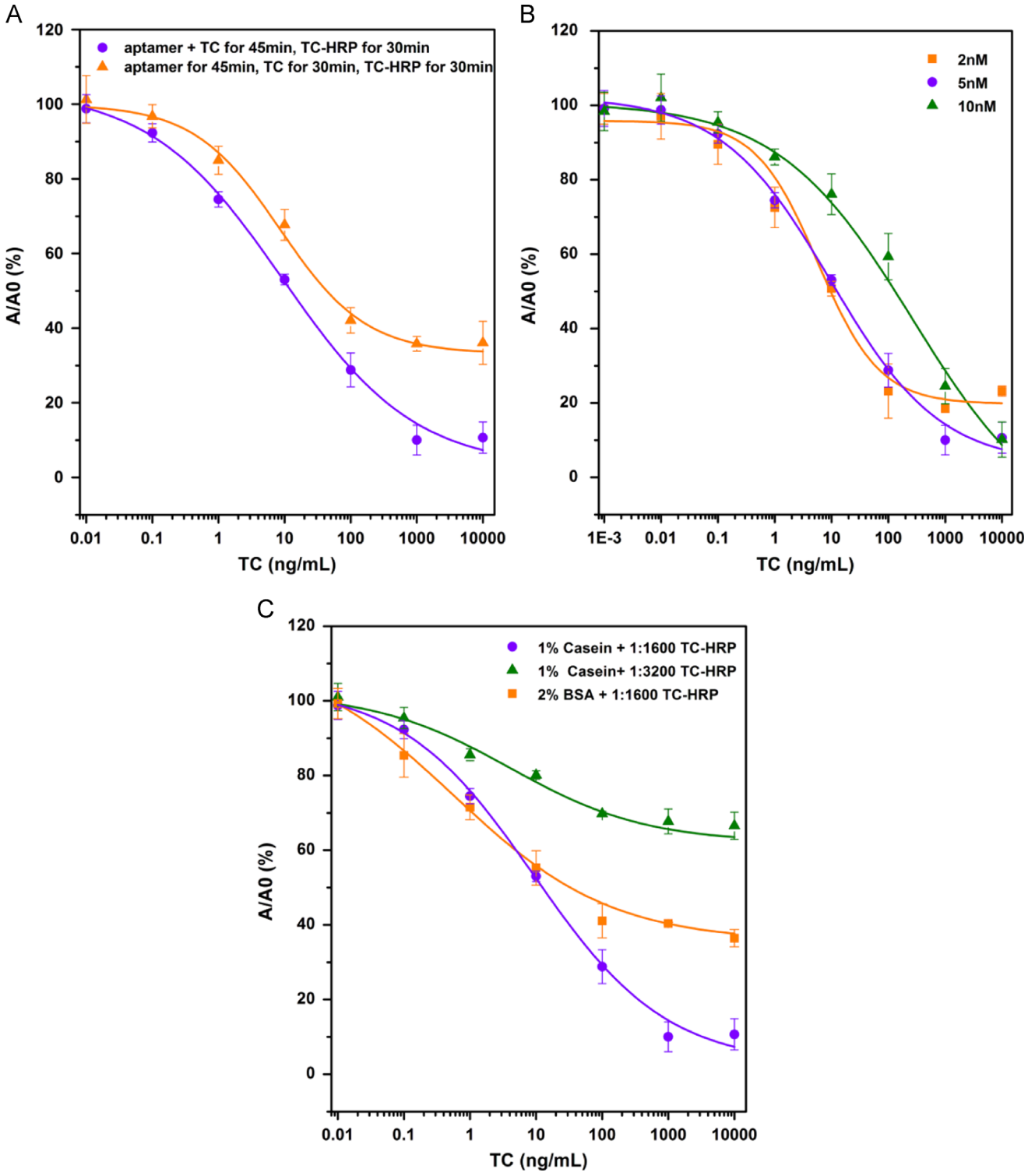

Fig. 4. Direct competitive ELAA in buffer. (A) Inhibition curves for TC by *dc*-ELAA using different operation procedure, with 5nM aptamer and 1:1600 TC–HRP. (B) Inhibition curves for TC by *dc*-ELAA using various concentrations of biotinylated aptamer (2, 5, 10 nM). (C) Inhibition curves for TC by *dc*-ELAA using various concentrations of TC–HRP or different blocking agent. Data points are the average plus ± one standard deviation ($n=3$).

was utilized to remove ions metals as Na_2EDTA has a greater affinity for cations than TC [42], and TCA was employed as acidic deproteination agent. Moreover, honey solutions were kept in the dark to keep TC stable in honey for subsequent detection, since TC is light-sensitive.

The resulting matrix-matched standard TC in honey solutions was used in this study. The dose-response curves were explored in accordance with the selected dose-response curve in buffer (with 5 nM aptamer in Fig. 4(B)), using 1 μg/mL SA, 5 nM aptamer, and 1:1600 TC–HRP. The competition in honey showed satisfactory inhibition curve (Fig. 5(A)). The dose-response curves in buffer and honey were calculated as following: (1) A/A_0 (%) of TC in buffer $= -20.71 \times \text{Log [TC]} + 73.31$, $R^2 = 0.9957$ (Fig. 5(B)); (2) A/A_0 (%) of TC in honey $= -21.83 \times \text{Log [TC]} + 80.75$, $R^2 = 0.9880$ (Fig. 5(B)).

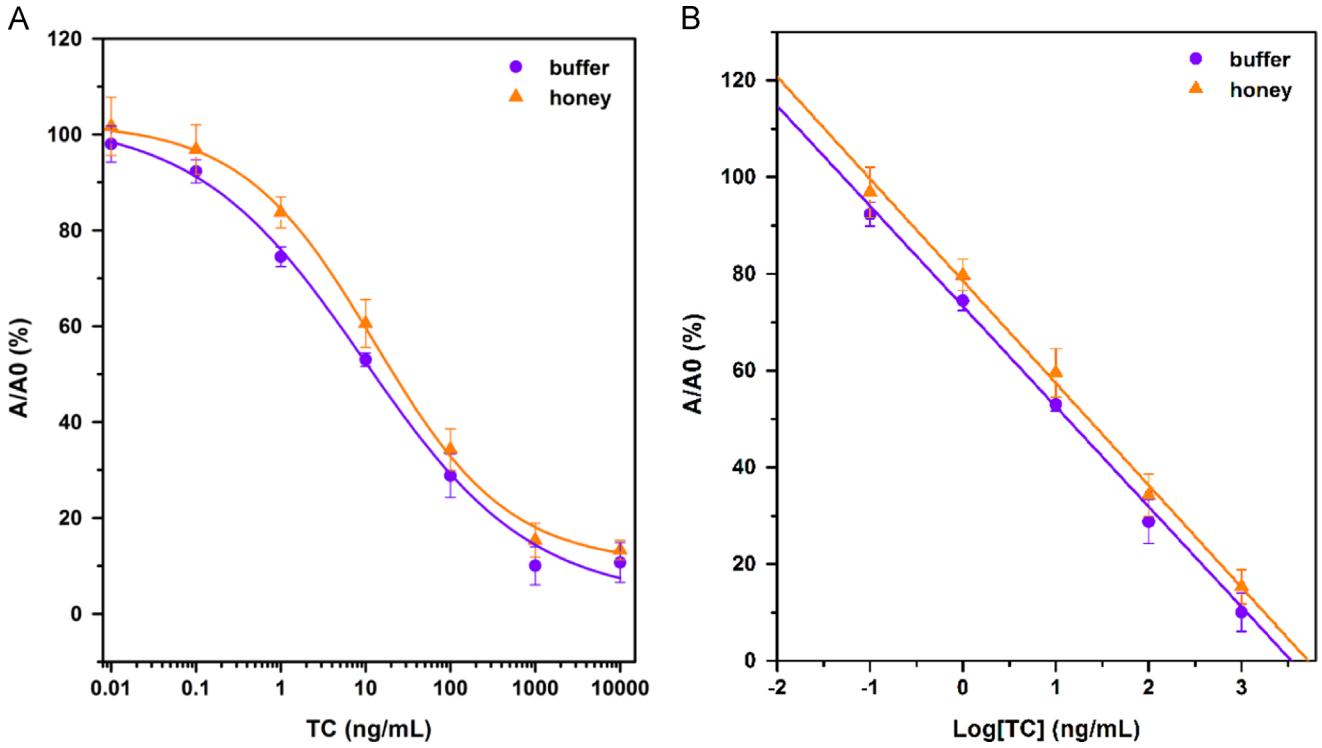

Fig. 5. Dose-response curves and calibration curves for TC. (A) Inhibition curves using 1 μg/mL of SA, 5 nM aptamer and 1:1600 TC–HRP in buffer and honey. (B) Calibration curve for TC in buffer and honey. Data points are the average plus ± one standard deviation (n=3).

Table 1
Comparison of methods reported for detection of TC in honey.

Analytical methods	LOD	Converted (ng/mL)[a]	Linearity (y=ax+b, a, b, r^2)	Linear range	Converted (ng/mL)[a]	References
dc-ELAA	9.78×10^{-2} ng/mL	0.0978	$y=-21.83x+80.75$, $r^2=0.9880$	0.1–1000 ng/mL	0.1–1000	
ELISA	3.98×10^{-10} M	0.17	$y=-0.446x-2.60$, $r^2=0.980$	$3.16–316\times10^{-9}$ M	1.4–140.4	[50]
ECL	2.0×10^{-12} g/mL	0.002	N/A	$4–400\times10^{-11}$ g/mL	0.04–4	[49]
FI-ECL	4.0×10^{-9} g/mL	4.0	$y=0.3140x+4.0997$, $r^2=0.9974$	$2–1000\times10^{-8}$ g/mL	20–10000	[51]
Dipsticks	7 μg/kg	7	N/A	N/A	N/A	[52]
CL	2.2×10^{-8} mol/L	9.78	$y=8.586\times10^{10}x+1.966\times10^{4}$, $r^2=0.9991$	$5.0–600\times10^{-8}$ mol/L	22.2–2666	[53]
HPLC	5.8 μg/kg	5.8	$y=0.25\ x+0.56$, $r^2=0.9998$	20.1–301.2 μg/kg	20.1–301.2	[54]
HPLC	0.12 ng/g	0.12	$y=474.2x+204.7$, $r^2=0.997$	1.0–125 ng/g	1.0–12	[55]
Fluorescence	0.3 μg/g	300	N/A	N/A	N/A	[56]
HPLC	8.3 μg/kg	8.3	N/A	N/A	N/A	[57]
TFC-UHPLC-Orbitrap- MS	5 μg/kg	5	N/A	5–250 μg/kg	N/A	[58]

N/A, not available/applicable

[a] Converted for easy comparison

A LOD of 0.0659 ng/mL and a linear range of 0.1–1000 ng/mL were achieved in buffer. Additionally, the LOD and the linear range were 0.0978 ng/mL and 0.1–1000 ng/mL respectively in honey. The results indicate that the *dc*-ELAA method in this assay yields similar LOD values in buffer and honey without obvious matrix effect.

Several studies have reported to select specific DNA or RNA aptamer for TC [34,43,44] and developed aptasensors to detect TC in food. Most of the aptasensors are electrochemical aptamer-based biosensors to detect TC residue in milk [45–48], and few studies are performed on microtiter plate platform which can achieve high-throughput detection [36,40]. In recent years, some other novel analytical methods were reported for the quantification of tetracycline residue in honey (Table 1). The *dc*-ELAA in the present study demonstrated remarkable advantages in terms of detection limit, linear range compared with the other reported rapid analytical methods.

3.4. Recovery and cross-reactivity study

The specificity of the 76mer aptamer employed in the aptasensor was evaluated for cross-reactivity against several common members of TCs (oxytetracycline, tetracycline, doxycycline) when selected by SELEX [34]. The reported results showed the 76mer ssDNA aptamer yield a measurable specific binding to TC than its structural similarities. What's more, the specificity and high affinity of the aptamer-analyte interaction significantly simplified sample pretreatment.

Accuracy of the aptasensor detecting TC in honey samples was evaluated by recovery rate (%) and data reproducibility (% relative standard deviation, RSD). Free TC (0.1 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL and 1000 ng/mL) were spiked in honey solution and mean percentage recoveries were analyzed to be 92.09–109.7%, the RSD was lower than 7% in honey (Table 2). To validate the *dc*-

Table 2
Recovery study of *dc*-ELAA and ELISA.

Methods	Spiked concentration (ng/mL)	Measured concentration (ng/mL, mean ± SD)	Recovery (%, mean)	RSD (%)
dc-ELAA	0.1	0.1097 ± 0.004993	109.7	4.550
	1	0.9209 ± 0.05963	92.09	6.475
	10	9.331 ± 0.5334	93.31	5.716
	100	97.48 ± 3.944	97.48	3.944
	1000	987.5 ± 25.13	98.75	2.544
ELISA	0.1	0.1118 ± 0.006785	111.8	6.066
	1	0.8656 ± 0.02987	86.56	3.451
	10	11.20 ± 0.4946	112.0	4.416
	100	95.25 ± 4.389	95.25	4.608
	1000	1021 ± 26.66	102.1	2.610

SD: standard deviation ($n=7$).
RSD: relative standard deviation.

Table 3
Detection by *dc*-ELAA, *ic*-ELAA, and ELISA.

Honeys	Measured concentration (ng/mL, mean ± SD)		
	dc-ELAA	*ic*-ELAA	ELISA
Acacia Honey	0.1782 ± 0.001604	0.1067 ± 0.07422	0.1239 ± 0.01808
Pipa Honey	1.594 ± 0.07190	1.583 ± 0.1416	1.931 ± 0.04943
Jujube Honey	1.157 ± 0.07660	1.683 ± 0.1445	1.629 ± 0.1448
Polyfloral Honey	1.745 ± 0.1063	1.65 ± 0.02065	2.119 ± 0.1576

SD: standard deviation ($n=7$).

ELAA, a standard ELISA was performed in the recovery study with the same spiked concentration of tetracycline in honey. As shown in Table 2, the results of the two methods demonstrate satisfactory consistency. The results enabled the use of *dc*-ELAA as satisfactory screening method.

3.5. Comparison of direct-competitive ELAA (dc-ELAA), indirect-competitive ELAA (ic-ELAA), and ELISA

As sandwich assay scheme is not available with TC detection owing to its small size (Mw 444.4), detection approaches based on competitive formats are preferred, performed directly or indirectly. An aptasensor based on *ic*-ELAA (indirect competitive enzyme-linked aptamer assay) scheme was developed in our previous study [36]. Analysis time of the two competitive methods was similar. The *dc*-ELAA in this study with a wider linear range can be used to detect higher concentration of TC in honey, thus applicable for countries with MRL more than 100 ng/mL, and is more practical for on-site detection. The *ic*-ELAA provided slightly higher sensitivity than the *dc*-ELAA in terms of LOD (0.0096 ng/mL in *ic*-ELAA) partly owing to the signal amplification of the indirect competition based on the highly specific biotin-streptavidin combination. In the indirect competitive aptasensor, SA–HRP (horseradish peroxidase labeled streptavidin) was introduced to amplify the signal after the competition of TC, TC–BSA for aptamer–biotin. And in the present study, SA-B was used to immobilize the aptamer on the microtiter plates, TC–HRP was employed as competitor and enzyme tracer, while there is no other amplification of competition. In short, the direct and indirect competitive aptasensors have their own merits, and they can both be employed to detect TC in honey.

In addition, the *dc*-ELAA was applied to quantify the TC residue in several collected honey samples (from white to dark, monofloral and polyfloral), as well as the *ic*-ELAA and a standard ELISA (A/A_0 (%)$=-39.45\times$ Log [TC]$+53.58$, $R^2=0.9905$). The results showed good proximity of the three methods (Table 3).

4. Conclusions

In conclusion, an improved direct competitive assay-based aptasensor for detection of tetracycline in honey was developed. In this study, detailed operating optimization was carried out and dose-response behavior was investigated both in buffer and honey samples. The optimized *dc*-ELAA showed a good limit of detection (LOD of 0.0978 ng/mL), a wide linear range (0.1–1000 ng/mL) and high recovery rates (92.09%–109.7% in TC spiked honey samples). The proposed method is promising to provide a simple and sensitive screening scheme for on-site quality surveillance of TC in honey and related ad hoc food safety issues.

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