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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.

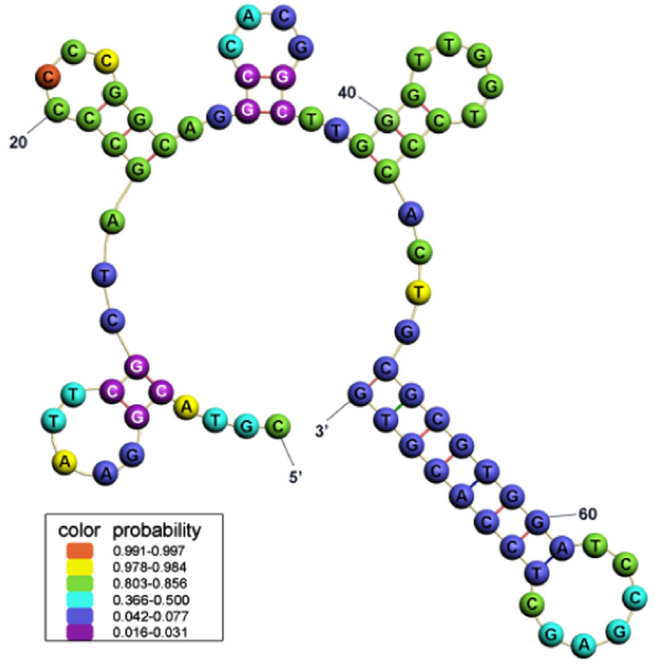

Fig. 1. Secondary structure of the 76mer ssDNA aptamer predicted by m-fold according to free energy minimization algorithm [36]. Color annotation means the probability.

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