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ORIGINAL ARTICLE

A Sensitive Fluorescent Assay for Tetracycline Detection Based on Triple-helix Aptamer Probe and Cyclodextrin Supramolecular Inclusion

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Abstract
Herein, an effective pyrene excimer signaled fluorescent biosensor for the determination of tetracycline based on triple-helix aptamer probe (TAP) and supramolecular inclusion of cyclodextrin was reported. The TAP was devised containing an aptamer loop, two DNA segment stems and a triplex-forming oligonucleotide (signal probe) labeled with pyrenes at 5′ and 3′ ends. The presence of target could result in its binding towards aptamer with a mighty affinity, leading to a conformation change of the TAP and whereupon the release of the signal probe. This liberty of signal probe enabled the formation of pyrene excimer, generating fluorescence signals. Further, signal amplification was fulfilled through the addition of γ-cyclodextrin which could interact with pyrene dimer, thus leading to an enhanced "on-state" of the sensing ensemble. In contrast, when the target was absent, the sensing ensemble remained "off-state" because of the long distance between two pyrene molecules. When the conditions were properly optimized, the increasing signal kept a linear dependence on target concentrations ranging from 5.0 nM to 100 nM, and the detection limit reached as low as 1.6 nM. In this way, a newly-constructed, simple, and economically affordable protocol enjoys desirable efficiency, sensitivity, specificity in biosensing. Also, its universality as another attractive behalf in assaying diverse targets was envisioned with only the need of matched aptamer replacement.

Keywords Tetracycline · Fluorescent · Biosensor · γ-cyclodextrin · Triple-helix aptamer probe

Introduction

When researches on the double strand DNA became increasingly thorough, scientists were having their eyes on other structures of DNA sequences and their intriguing possibilities. Triple-helix aptamer probe (TAP) was born at certain expectation which could be formed autonomously relying on Watson-Crick and Hoogsteen base parings under optimized conditions [1, 2]. In structure of stem-loop, it was composed of an aptamer sequence, two arm-like DNA segments flanked by sides and a triplex formed oligonucleotide. Apart from advantage of novelty, compared with routine double strand DNA, TAP were equally structurally stable but significantly sensitive and specific towards targets due to the affinity realized by the aptamer loop. Furthermore, the versatility could be attained owing to the flexibility of aptamer alteration with no need to redesign the probes functioned as signal transmitters, enabling its potential popularization in assaying diverse targets. As thus, the TAP was appealing enough to be introduced to the construction of a series of bio-sensing strategies [3–5]. In the function of TAP system, aptamers, generally referring to a single strand DNA or RNA separated from libraries randomly via famous systematic Evolution of Ligands by Exponential Enrichment SELEX, were deemed to be powerful recognition tools because of strong binding affinities in biosensing applications for multitudinous targets ranging from molecules, proteins to even cells [6–8].

Tetracycline as one of most typical representatives in antibiotics was initially discovered in culture medium of streptomyces aureofaciens and streptomyces rimosus in as early as 1940s. It thereupon obtained positive reputation bolstered by

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virtues of favorable water solubility, stable chemical properties, inexpensiveness, and broad-spectrum antibiotic capabilities, which made itself a preferred option for bacteria-infectious disease treatments and more recently- as a common additive substantially exploited for animal husbandry. With years of accumulation, tetracycline residues happened in these animal derived foods, thus exerting a detrimental impact on their consumers. With a long-term exposure to tetracycline, human bodies were chronically poisoned and likely to develop organ lesions including intestines, stomach, and liver etc. [9]. Besides, Pregnant women and children were extraordinarily allergic to tetracycline- by even limited amounts of which children could be interfered in the absorption of Calcium and subjected to dental diseases such as tetracycline pigmentation teeth [10]. Statistic manifested that excessive exposure to tetracycline in pregnancy increased steeply the teratogenicity rate [11]. Therefore, it is highly urgent to devise a cogent method with satisfactory sensitivity, specificity, rapidity and efficiency for the quantitative monitoring the contamination of tetracycline.

To date, assorted approaches were employed for tetracycline detection covering fluorescent assay [12], enzyme-associated immunosorbent assay [13], electrochemical assay [14, 15], colorimetric assay [16, 17], chemiluminescence [18, 19], thin-layer chromatography [20], high-performance liquid chromatography [21], surface enhanced Raman scattering [22] and photoelectrochemical assay [23, 24] etc. Admittedly, considerable significantly as these methods progressed, they were somewhat discounted by intrinsic drawbacks involving the undesirable specificity and selectivity caused by the structural similarities between tetracyclines, tediousness and time consumption, heavy dependence on complicated or/and costly experimental instruments and demanding sophisticated operators which restrained their popularization in on-site/ real-time and effective quantification of tetracycline. To circumvent these as-mentioned problems, fluorescence probe assay became vitally applicable option and captured plentiful imagination bolster by virtues of satisfying sensitivity, selectivity, operative simplicity, affordable instruments and device miniaturization.

Excimer probes were considered to be appealing conformations relying on the large Stokes shift and long-life fluorescence of pyrene excimer molecules [25–27]. The pyrene molecules were modified upon both termini of a DNA sequence and under certain conditions, the both ends were able to get into proximity, producing an excimer. In comparison with the traditional molecular beacon, the pyrene excimer with sustainable lifetime enabled the fluorescence detection to be accomplished even in a complex cell. Nevertheless, excimer formation depended heavenly on the distance and geometry, requiring intimate physical contact between the monomers and meanwhile a cooperative alignment of transition dipoles, which thus was easily interfered in. The participation of γ-cyclodextrin in the sensing ensemble provided a solution to this problem. It was because that the establishment of the host–guest complex between the pyrene dimer and γ-cyclodextrin could for one hand protect the pyrene dimer from the quenching effect by environmental disturbance, promoting the microenvironment for the pyrene dimer and considerably increasing the fluorescence intensity of the pyrene excimer, but for the other hand enabled the fluorescent lifetime of the pyrene excimer more sustainable than that of the pyrene dimer alone. Hence the pyrene excimer could be produced via the function of the γ-cyclodextrin inclusion, on the basis of which a visible pyrene excimer emission was obtained [28–30].

Inspired by as-mentioned respects, we envisioned an interesting biosensor coupling the specialty of the TAP with the pyrene excimer molecules in the light of fluorescence probe assay for the sensitive detection of tetracycline. Firstly, TAP was fabricated with two segments stems, an aptamer loop, and a triplex-forming oligonucleotide (signal probe) modified with bis-pyrenes at both ends of 5' and 3'. Subsequently, the target got involved, resulting in the structural changes of the TAP due to the binding event between aptamer loop and target, and thus causing the departure of the signal probe from TAP. Next, the formation pyrene excimer was fulfilled arisen from this release of signal probe, yielding fluorescence signals. Moreover, the signals were further amplified by means of adding γ-cyclodextrin because the host - guest complex formed could stabilize the performance of the pyrene excimer and therefore remarkably increase the fluorescence intensity. Also, one superiority was also expected that owing to the long-standing lifetime of the pyrene excimer fluorescence than those of common fluorescent components, this fluorescence protocol was thus outlooked for application in complicated biological real samples.

Experimental Section

Reagents and Materials

Tetracycline, oxytetracycline, chloramphenicol, kanamycin and norfloxacin were purchased from Aladdin Industrial Corporation (Shanghai, China). The chemical structure of the antibiotics was shown in Fig. 1. Trichloroacetic acid, chloroform, α-, ß- and γ-cyclodextrins were bought from Beijing Chemical Reagent Company (Beijing, China). All chemical reagents were analytical grade and were used as received without further purification. The ultrapure water used throughout all experiments was purified by a Milli-Q system (Millipore, Bedford, MA, USA). The antibiotic stock solution was prepared by ultrapure water and stored at 4 in refrigerator. The raw liquid milk was purchased from the local supermarket. 10 mM Phosphate buffer solution (PBS, pH 6.3) containing

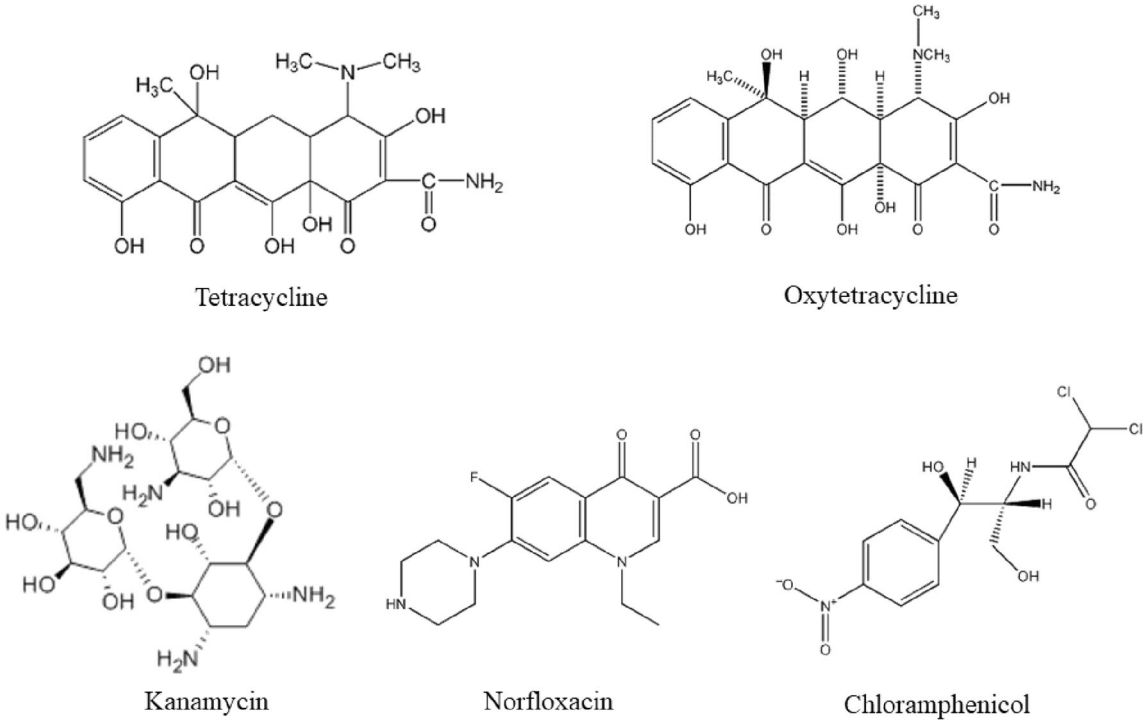


Fig. 1 The chemical structure of the antibiotics

20 mM KCl and 2.5 mM $MgCl_2$ was used for homogeneous fluorescent detection of tetracycline.

Oligonucleotides used in this work were synthesized by Takara Bio Inc (Dalian,China). All DNA sequences were displayed in Table 1 and prepared with ultrapure water as stock solutions stored at -20 °C before use, the concentrations of the DNA solution were quantified by UV absorption. Playing the role of realizing the signal transmission, pyrene molecules (Py) were modified on 5' end and 3' end of signal probe (SP) respectively. Three aptamer sequences (A-8, A-40, and A-76) purified by HPLC and each aptamer was extended with two arm segments containing 7 bases of T and C nucleotides at each end.

Preparation of TAP Complex

All the oligonucleotides stock solutions were diluted by PBS and sequentially heated at 90 °C for 6 min to reduce intermolecular interactions, the oligonucleotides solutions were then cooled slowly to room temperature. Next, 50 μL 10 μM signal probe and 50 μL 10 μM aptamer flanked by two arm segments (A-8, A-76, and A-40) were mixed in a 400 μL PBS for the assembling of TAP complex before being incubated at 30 for hybridizing them with each other. 30 min was the optimal time to guarantee that TAP was completely formed. Subsequently, the TAP-8, TAP-76, and TAP-40 complex solution was prepared (the final concentration was 1.0 μM).

General Procedure for Fluorescence Biosensing of Tetracycline

In a typical experiment, 50 μL different tetracycline concentrations stock solutions were added into 1.5 mL centrifuge tubes respectively, and vortex-mixed at room temperature. Then, 50 μL 1.0 μM TAP-8 was added, vortex-mixed thoroughly and diluted to 450 μL with PBS buffer solution. To obtain the enhanced fluorescence signals, a series concentration of γ-cyclodextrin were involved in the preparation of

Table 1 Oligonucleotide sequences used in this work

Name	Sequence(from 5'-3')
SP	Py-GAGGAGAGAGAGAGATCCTC-Py
A-8	CTCTCTC **CGGTGGTG** CTCTCTC
A-76	CTCTCTC **CGTACG GAATTCGCTAGCCCC CCG GCA GGC CAC GGC TTG GGT TGG TCCCAC TGC GCG TGG ATC CGA GCT CCA CGT G** CTCTCTC
A-40	CTCTCTC **GTT TGT GTA TTA CAG TTA TGT TAC CCT CAT TTT TCT GAAC** CTCTCTC

reaction solutions (50 μL). Sequentially, fluorescence measurements were recorded by a Synergy H1 Hybrid Multi-Mode Microplate Reader spectrofluorometer (BioTek's Hybrid Technology, USA), whose emission spectra was set up in the range of 360–700 nm in a 96-well plate. The maximum fluorescence intensity at 485 nm was setting to detection of tetracycline by exciting at 345 nm. Slits for both the excitation and the emission were set at 5 nm. Every step of this assay was independently repeated more than five times. Taking the TAP complex solution without tetracycline as control, the fluorescence emission intensity at 485 nm was proportional to the tetracycline concentration, in which F0 and F1 were the fluorescence emission signal of the reaction solution in the absence and presence of tetracycline, respectively. The fluorescence intensity of the TAP complex was measured at room temperature. The same procedures were performed in the presence of oxytetracycline, chloramphenicol, kanamycin and norfloxacin instead of tetracycline to assess the selectivity against other antibiotics.

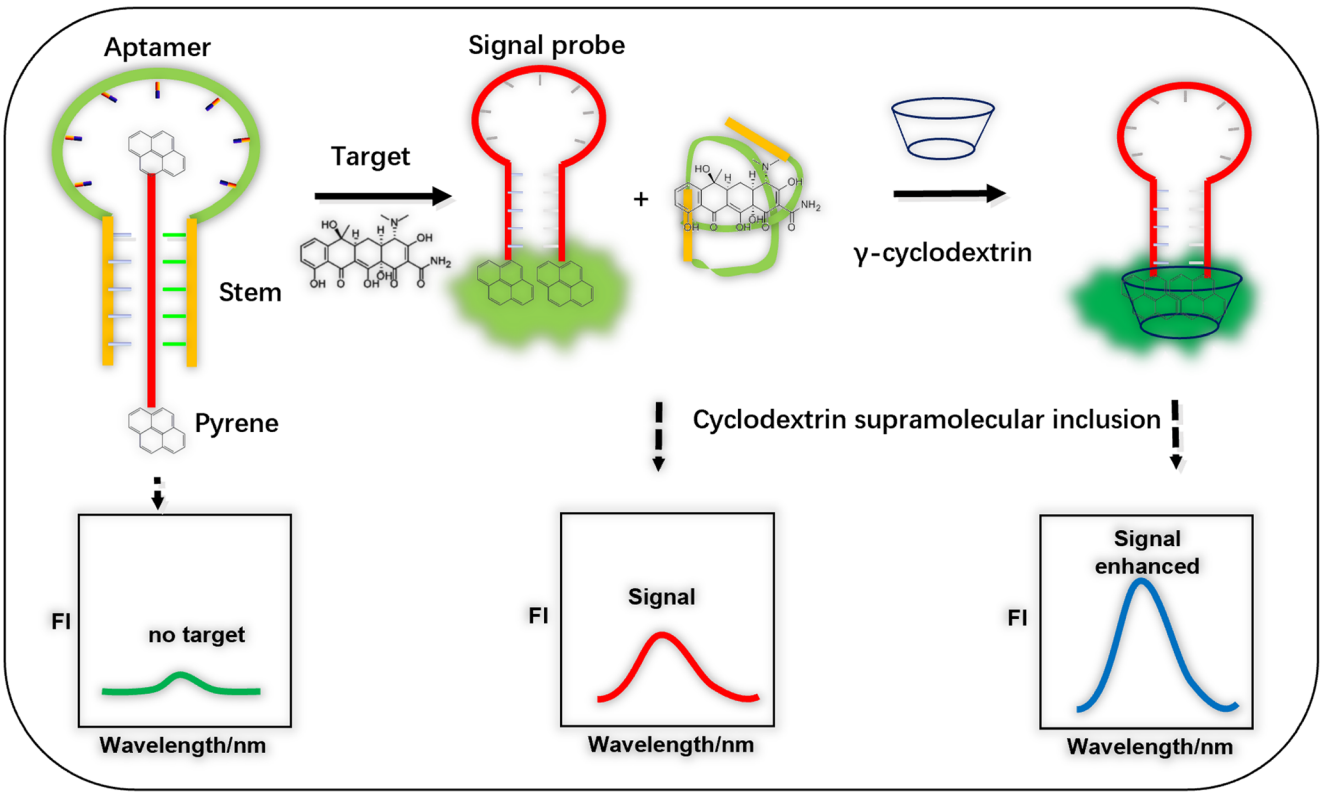
Fluorescence Detection of Tetracycline in Milk Samples

The pretreatment procedure for the raw milk samples was carried out according to the reference [31], with the brief introduction was as follows. Firstly, for removing the proteins, organic substances and fats, 2 mL raw milk was added into a 15 mL centrifuge tube, and diluted with ultrapure water to 10 mL, the next, 2 mL chloroform and 2 mL 10% trichloroacetic acid were added in the centrifuge tube. The mixture was then ultrasonically treated for 10 min and centrifuged at 15,000 rpm for 10 min to separate the deposit. Secondly, the collected supernatant was transferred into another centrifuge tube and centrifuged at 15,000 rpm for 10 min to remove the deposit once again and get the final supernatant. Finally, the obtained supernatant was diluted 20 times for detection. A different concentration of tetracyclines was spiked into the treated raw milk sample, then analyzed in accordance with the same procedure.

Results and Discussion

Principle of Sensing Platform

The sensor was started from a TAP formation on the basis of Watson-Crick and Hoogsteen base pairings between two arm segments, an aptamer loop and signal probe (SP) with pyrene molecules labeled at its both 5′ and 3′ terminus. As exhibited in Scheme 1, the sensing system remained "off-state" due to the conservation of a more stable TAP that kept a long distance between the two modified pyrene molecules under the



Scheme 1 The principle of the fluorescent assay for quantitative detection of tetracycline based on triple-helix aptamer probe and γ-cyclodextrin supramolecular inclusion

absence of tetracycline. Nevertheless, after addition of tetracycline, the TAP complex was disassembled and the SP designed as a structure of molecular beacon was estranged. Thus, the pyrene excimer was generated by the self-complementary 5′ and 3′ ends of the SP, capable of yielding fluorescence signals. Further, the participation of γ-cyclodextrin safeguarded the positive interaction of pyrene dimer, thus significantly promoting the pyrene excimer emission and the fluorescence intensity (on-state). In a word, the target presented dismounted the TAP, liberated the SP, and produced a strong fluorescence signal. To be noted, this protocol was well-performed owing to two contributors as followed: for one hand, it adopted TAP, possessing aptamer as its loop, which could capture and bind with target efficiently, therefore strengthening remarkably the sensitivity and specificity of the whole ensemble. For the other hand, the participation of γ-cyclodextrin could render protection for the emission of pyrene dimer from the quenching effect of environmental conditions, amplifying considerably the fluorescence intensity of the pyrene excimer. Thus, the target quantification could be realized in line with the enhancement of the fluorescence intensity and the "signal-on" fluorescence strategy was accomplished for the sane determination of tetracycline.

The Feasibility and Signal Amplification of the Assay

The feasibility of the assay was examined by fluorescence emission spectra of TAP-8 complex under various conditions (Fig. 2). The fluorescence spectra of A-8 and TAP-8 (A-8 + SP) were compared without tetracycline involvement, which were nearly nonfluorescent (curves a and curves b). It could be seen that there was a steep significant rise in fluorescence intensity at 485 nm in the presence of tetracycline (curve c). The capability of signal amplification accomplished by γ-cyclodextrin was tested through recording fluorescence signal fluctuation before and after the addition of γ- cyclodextrin. Compared with curve c, a notable and stronger signal readout was observed after the participation of γ- cyclodextrin because the γ- cyclodextrin fulfilled the inclusion of pyrene dimer, strengthening their positive interaction, guaranteeing their emission and thus significantly enhancing the signal intensity (curve d). In this way, a conclusion was worked out that the signal amplification function of γ- cyclodextrin for the sensing system was arisen from housing some pyrene dimer within the γ- cyclodextrin cavity to make the two pyrene molecules closer, triggering apparent excimer fluorescence enhancement when target was added. These above results verified the feasibility that the target could trigger the fluorescence increased and enhanced through the assistance of γ-cyclodextrin, giving rise to a "signal-on" fluorescence of the sensing system for the measurement of tetracycline.

The Selection of Cyclodextrin

Containing six, seven, and eight glucose residues respectively, α-, β-, and γ-cyclodextrins possessed lipophilic cavities with diverse inner diameters which enabled them to form host-guest inclusion complexes with ions, molecules and etc. Albeit in aqueous solutions the stoichiometry of the formation towards complex was commonly 1:1, yet inclusion complexes of "one host-two guests" were deemed to be proper needed in this experiment enjoying large cavity. Pyrene was acknowledged as a classical aromatic compound performing excimer fluorescence due to collisional interaction between excited and ground-state monomers. Hence the fluorescence spectra

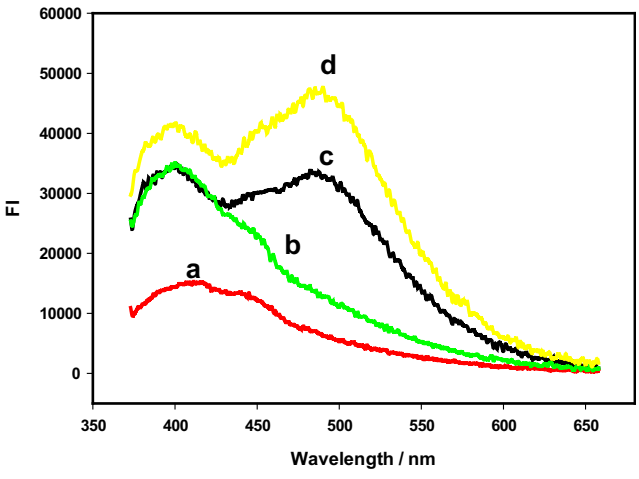


Fig. 2 Fluorescence emission spectra of the sensing system under different conditions. In the absence (curve a) and presence of target (curve b), and further addition of γ-cyclodextrin (curve c), curve d was the fluorescence emission spectra in the presence of γ-cyclodextrin

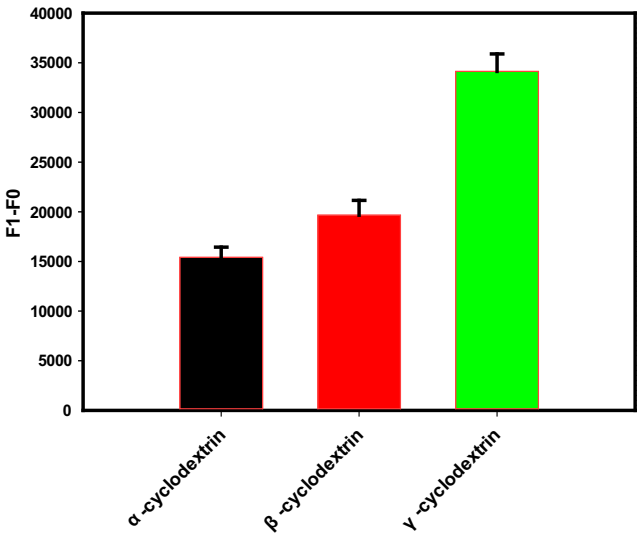


Fig. 3 The fluorescence signal enhancement of α-, β-, and γ-cyclodextrins

of pyrene in aqueous α-, ß-, and γ-cyclodextrin solutions were observed and compared. As exhibited in Fig. 3, much more intensely did the excimer fluorescence of pyrene manifest in aqueous γ-cyclodextrin solutions compared with those of the other two solutions, which thus demonstrating that the pyrene molecules were covered forming a dimer in the γ-cyclodextrin cavity having a perfect size of cavity for inclusion. Therefore, γ-cyclodextrin was selected to enhance fluorescence signals in this work.

Optimization of Experimental Conditions

On purpose of optimizing the experimental conditions for tetracycline detection, the influence by several vital parameters concerning detection was investigated, including the aptamer length of TAP, the concentration of TAP-8, the concentration of γ-cyclodextrin and the incubating time of tetracycline with TAP-8. In the TAP, the aptamer (loop sequences) and two arms (stem sequences) were designed to match the SP to form a triplex motif through T•A-T and C^+•G-C base triplets. As mentioned in introduction, because there could be a possibility of existence of several aptamer towards the same target with discriminatory affinities, thus the lengths of aptamer were investigated. In this work, three aptamers with different lengths were designed to achieve the purpose of detection (A-8, A-76, A-40, the sequences were shown in Table 1). After binding with SP and stems, the TAP-8, TAP-76, and TAP-40 corresponding to 8, 76 and 40 bases were formed and the effects of these sequences on the formation of TAP complex were studied. It revealed that the TAP-8 could generate strong fluorescence in the presence of target, as shown in Fig. 4a. After replacing A8 with A-76 and A-40 respectively, the fluorescence emission slightly decreased. Namely, the TAP assembled by A-8, A-76 and A-40 manifested nearly equally favorable fluorescence intensity in the presence of target. However, in terms of the advantages of A-8 which needed the fewest bases for the establishment of TAP but even yielded better fluorescence emission, the TAP-8 was chosen for the following experiments.

Playing an essential role in the stability insurance of the Hoogsteen base pairing, the pH which exerted an appreciable influence on the formation of TAP was thus investigated. Taking this factor into consideration, as depicted in Fig. 4b, when the pH increased, the fluorescence intensity of the

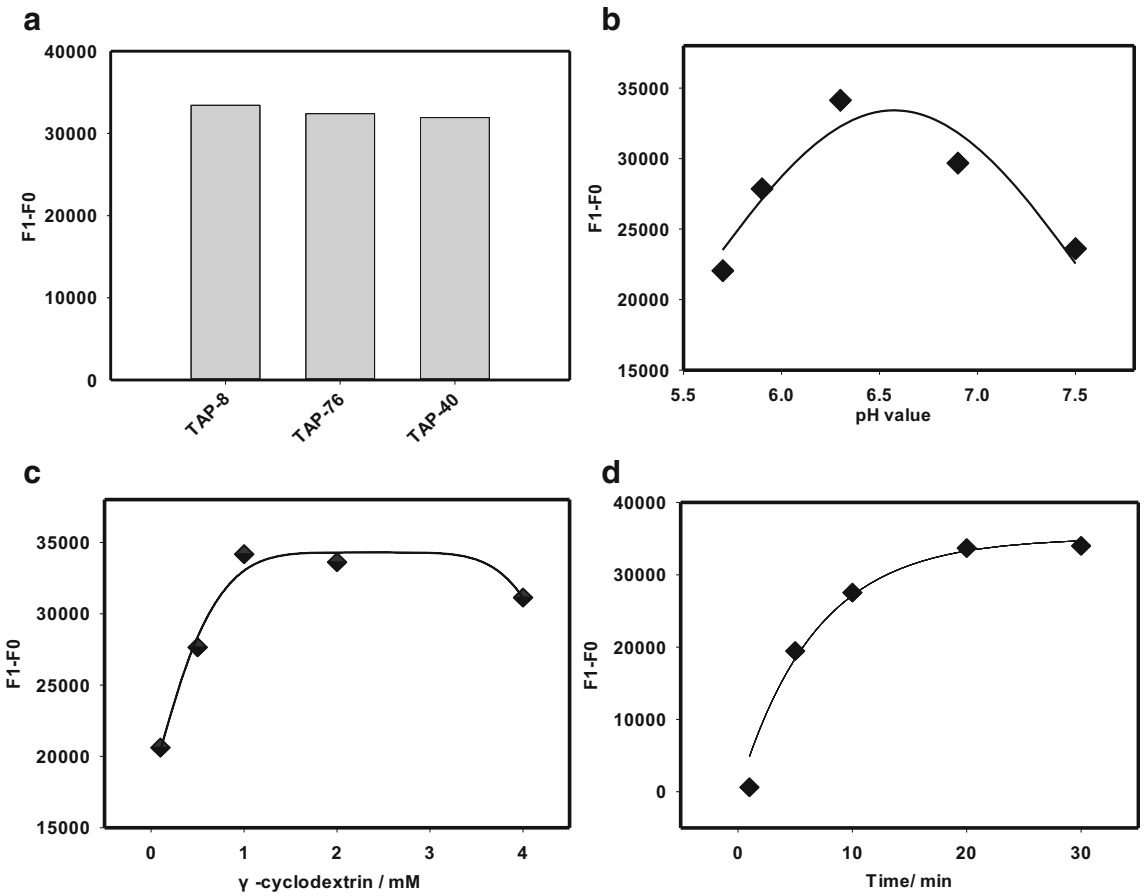


Fig. 4 Effects of (**a**) the aptamer length of TAP, (**b**) the pH value, (**c**) the concentration of γ-cyclodextrin, (**d**) the incubating time of tetracycline with TAP-8. F1 and F0 were the fluorescence intensities of sensing system in the presence and absence of target, respectively. All assays were carried out in 10 mM PBS with 100 nM tetracycline, 100 nM TAP-8 while the other parameters were under their optimal conditions

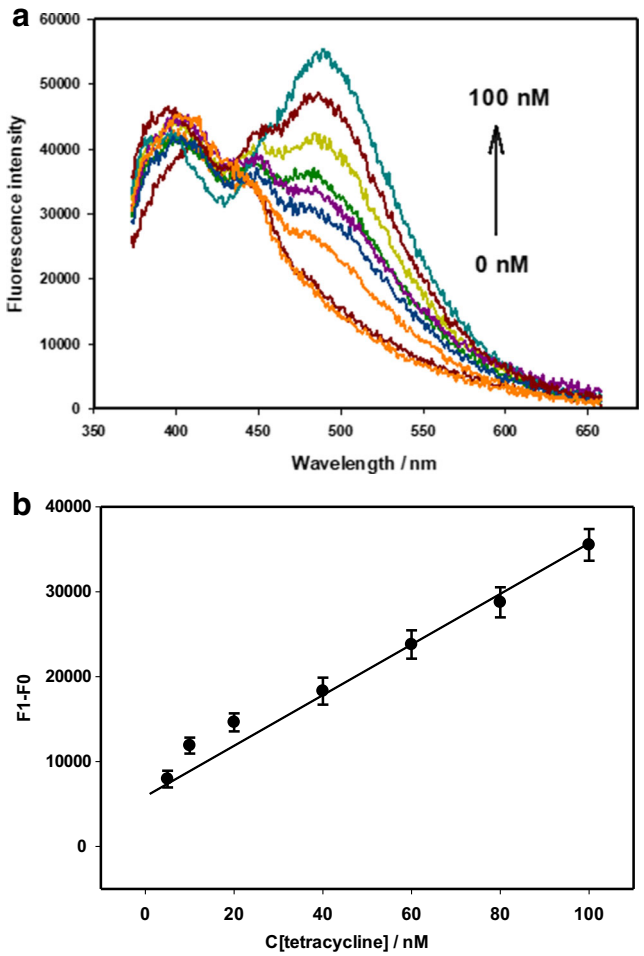


Fig. 5 **a** Fluorescence emission spectra of sensing platform in response of tetracycline at 0, 1, 5, 10, 20, 40, 60, 80, 100 nM. **b** Calibration curve for tetracycline detection (5, 10, 20, 40, 60, 80, 100 nM)

sensor got initially stronger and then weaker, peaking the response at pH of 6.3. It was primarily because that in acidic conditions, the protonation of cytosine residues assisted in the formation of Hoogsteen base pairing between T•A-T and C+•G-C triplets, reinforcing the virtuous interaction between the loop segments of the aptamer and the SP. And when the pH got higher, the imino groups of cytosines could not accomplish their protonation which made the Hoogsteen base pairing unstable. Hence pH 6.3 was fixed as an ideal value for the whole experiment.

In terms of fulfilling the optimal performance, the different concentrations of γ-cyclodextrins were studied because its concentrations affected the efficiency for fluorescence enhancement. As shown in Fig. 4c, the fluorescence intensity (F1-F0) strengthened and subsequently came to plateau as the concentration of γ-cyclodextrins increased. The results demonstrated that 1.0 mM γ-cyclodextrin was sufficient to increase the fluorescence intensity. Therefore, the corresponding concentration of γ-cyclodextrins (1.0 mM) was selected for the following experiments as the optimal value.

The effects of reaction time of tetracycline with TAP on the fluorescence intensity were investigated. As seen from Fig. 4d, the fluorescence intensity of F1-F0 solutions increased from 0 to 30 min, and then remained constant which indicated that the TAP fully reacted with tetracycline within a short time. Thus, 20 min was used as the optimal reaction time of tetracycline with the TAP.

Sensitivity and Specificity

The sensitivity of the as-constructed fluorescence assay for tetracycline was evaluated via testing a range of concentrations of tetracycline in the optimized experimental conditions. As exhibited in Fig. 5, although the concentrations of tetracycline from 1.0 nM to 100 nM were tested using this assay, but only in the range from 5.0 nM to 100 nM of the increasing tetracycline concentrations could maintain a linear increase of the fluorescence intensity. The dependent linear equation was $F1\text{-}F0 = 267.58\ C_{\text{tetracycline}} + 8071.5$ (R^2=0.9892), indicating that tetracycline concentration could be quantitatively derived. The detection limit, referring to the International Union of Pure and Applied Chemistry (IUPAC) standard (defined as 3 s/slope, where s was the relative standard deviation of a

Table 2 Comparison of different assays for tetracycline sensing

Sensing element	Technique	LOD (nM)	Linear range (nM)	Ref
Fe_3O_4 nanoparticles	Colorimetric	45	10-1000	[32]
Gold nanoparticles	Colorimetric	45.8	10–400	[33]
M-shape aptamer	Electrochemical	0.45	1.5–3500	[34]
Prussian blue-chitosan-glutaraldehyde	Electrochemical	0.32	$1.0–1.0 \times 10^7$	[35]
Nucleotide/lanthanide coordination polymer	Fluorescent	60	$100\text{-}2.0 \times 10^4$	[36]
Molecularly imprinted polymers/carbon quantum dots	Fluorescent	5.48	20-14000	[37]
Triple-helix aptamer probe/cyclodextrin	Fluorescent	1.6	5.0- 100	This work

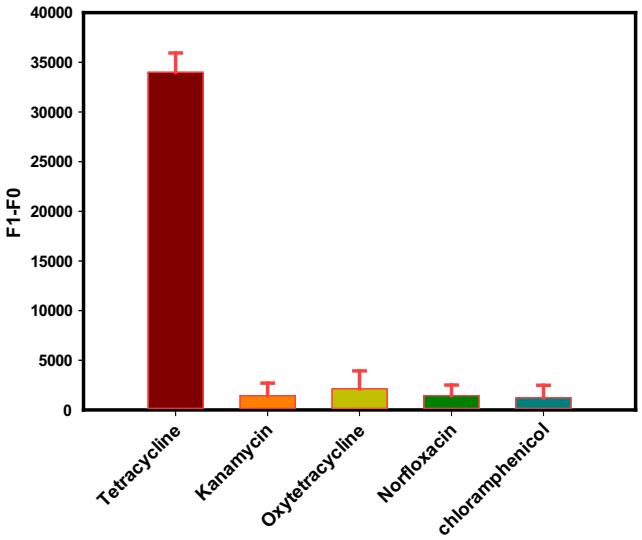


Fig. 6 Fluorescence intensity changes of the assay toward tetracycline and other antibiotics under the same condition. The concentrations of tetracycline, oxytetracycline, chloramphenicol, kanamycin and norfloxacin were 100 nM, respectively

blank solution, n = 5), was calculated to be 1.6 nM. Apparently, compared to other reported assays (Table 2), this method had a desirable sensitivity towards tetracycline even though the detection limit of the proposed strategy did not reach the bottom among the reported work. However, this approach realized the first attempt to couple TAP with γ-cyclodextrins for the sensitive fluorescent detection of tetracycline, which presented favorable advantages, covering good stability, outstanding sensitivity, and maintenance of the affinity and specificity induced by aptamer. Moreover, it was simple and economical which merely demanded one-step mixing and reaction to accomplish experimental process, and also considerably time-saving and convenient that could be completed within 50 min at room temperature including preparation of TAP complex.

In order to assess the specificity of the proposed protocol, the tetracycline and other four antibiotics substances (oxytetracycline, chloramphenicol, kanamycin and norfloxacin) at the same concentration were introduced to the platform whose responses were observed and investigated. As seen from Fig. 6, the rest substances did not yield any effective fluorescence intensity variations, manifesting that the strategy was remarkably selective and exclusive for the detection of tetracycline compared with other antibiotics. The satisfactory selectivity attributed to the reasons that aptamer in TAP-8 identified tiny conformational distinctions between target and other analogs. In this way, this technique with high sensitivity and selectivity was feasible to distinguish exactly tetracycline and quantify it in real samples.

Detection of Tetracycline in Raw Milk Samples

In real samples of raw milk, the fluorescent assay was challenged for the assay of tetracycline. The collected milk samples were pretreated at first. The samples were spiked with target was used for the determinations of tetracycline. Samples containing 10, 50 and 100 nM tetracycline were added into the final supernatant of raw milk Afterwards, the acquired samples spiked were investigated based on the procedure planed. The recovery ranged from 94.1%-102.5% with RSD (n = 5) lower than 5.0%, demonstrating that the constituents coexisted could scarcely result in appreciable interferences during the detection process towards the targets. Hence this method was convinced to be utilized for tracing tetracycline in raw milk.

Conclusions

In summary, a TAP and γ-cyclodextrin supramolecular inclusion-based sensing platform was successfully constructed for the homogeneous fluorescent detection of tetracycline. This aptamer-based TAP design conserved the selectivity and affinity of aptamers towards the target, making the platform precisely measure the tetracycline with a broad linear range and a desirable detection limit. Besides, it possessed some merits such as being operation-friendly, economic-effective and one more -universality realized only by altering the aptamer sequence but needing no redesign the signal probes. In this way, this as-constructed sensor was profoundly potential applied in the quantification and control of other toxic and harmful substances in food and environment fields.

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