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A label-free aptasensor for the detection of tetracycline based on the luminescence of SYBR Green I

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Abstract: A novel fluorescent method based on tetracycline-binding aptamers and the luminescence of SYBR Green I (SGI) was established for the sensitive and selective detection of tetracycline. Under native conditions, the aptamers of tetracycline show the G-quadruplex spatial structures while SGI is nearly nonfluorescent in aqueous solution. After mixture with the G-quadruplex structured aptamers, SGI can recognize and intercalate into the aptamers, resulting in a strong fluorescence emission. When the target tetracycline was added into the solution, the specific recognition and high-affinity binding of aptamers with tetracycline will induce the conformational changes of aptamers from G-quadruplex structures to hairpin structures. Thereafter, SGI will be released from the aptamer molecules, leading to the fluorescence decline. The quantitative detection of tetracycline can be achieved by measuring the fluorescence change of the system. Under the optimum conditions, the linear range of tetracycline in the milk was from 5 to 25 μg/mL, and the detection limit was as low as 0.10 μg/mL. The recoveries of the spiked milk samples were in the range of 98.98%-104.67% with the relative standard deviations (RSDs) of 0.16%-0.67%, and the results were in agreement with those from HPLC. Therefore, the biosensor based on the specific recognition of aptamers and the fluorescence properties of SGI can detect the tetracycline in milk accurately, rapidly and specifically.

Keywords: Tetracycline; Aptamers; SYBR Green I; Fluorescence

1. Introduction

As a kind of broad-spectrum antimicrobial drugs, tetracycline is widely used to treat bacterial infections in humans and animals because of its good antibacterial properties, low cost and small side effects. It also can promote the growth and reproduction of animals [1]. The widespread

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applicaions of tetracycline have led to excessive amounts of residues in daily foods such as milk [2], meat [3], honey [4], fish and eggs [5]. Tetracycline accumulated in dairy products, livestock and soil can be transmitted to the human body through the food chain. As tetracycline accumulates in the body, they could be harmful, for example, bacterial resistance can weaken the body's immune system. For allergic constitutions, tetracycline accumulation will cause allergic or toxic reactions, even inhibition of bone growth [6]. For the protection of human bodies, many countries and groups have set maximum residue limits (MRL) of tetracycline in food. For instance, the World Health Organization (WHO), European Union (EU) and Chinese Ministry of Agriculture have established the MRL of 0.10 mg/kg in milk for tetracycline, oxytetracycline and chlortetracycline [7].

Therefore, it is particularly important to develop a sensitive and effective method for the rapid detection of tetracycline. At present, the commonly used detection methods for tetracyclines are high performance liquid chromatography (HPLC) [8], liquid chromatography-mass spectrometry (LC-MS) [9,10], capillary electrophoresis (CE) [11,12], enzyme-linked immunosorbent assays (ELISA) [13] and chemiluminescence method [14,15], etc. However, many of them require time-consuming preprocessing steps and expensive equipments. HPLC, CE, and LC-MS usually need to use organic solvents that are harmful to health and the environment, and the extraction process of targets takes long time. Immunoassay methods are simple, specific and sensitive, but the preparation of antibodies takes a long time and immunization may appear false-positive results due to potential cross-reactivity with similar chemicals [16].

To overcome the limitations of the traditional methods described above, a number of aptamer-based tetracycline assays have been developed. Aptamers are functional DNAs or RNAs that are screened and amplified in vitro by systematic evolution of ligands by exponential enrichment (SELEX) and can selectively recognize and bind to small molecules, heavy metals, proteins, cells and even intact viral particles [17-23]. Aptamers have high affinity and specificity to their target molecules, similar to antigen-antibody interaction. However, compared to antibodies, they possess several advantageous features, such as easy production, chemical stability and flexibility, and particularly target versatility [24,25]. So far, analytical methods utilizing aptamers

as recognition elements have been increasingly used in the fields of biology, environmental hygiene, food safety monitoring, and so on [26-29]. Due to their own characteristics and advantages, aptamers have great potential in food safety control and monitoring. Antibiotic residues in animal-derived foods are very complex, and biosensors that use nucleic acid aptamers as recognition elements provide new insights into the detection and analysis of antibiotics. The sequences for tetracycline-binding aptamers have been screened [30]. Since then, several related researches have been reported on the aptasensors for the determination of tetracycline utilizing different signal transduction strategies such as colorimetry [31], fluorescence [32], chemiluminescence [33], electrochemical [34], photoelectrochemical techniques [35], and so on. Among the reported various aptasensors, fluorescent aptasensors have witnessed very rapid development with the advantages of simplicity, convenient signal transduction, easy operation and quick response, which can meet the requirements for detection of trace harmful components in food. For the labeled fluorescent aptasensors, covalent labeling of aptamers with fluorophores is time-consuming, labor-intensive and costly, furthermore it may decrease the binding affinity and selectivity of aptamers [36]. Obviously, label-free fluorescent aptasensors, which use DNA intercalation dyes such as SYBR Green I [37] and thiazole orange [38] as the signal reporters, can overcome these challenges.

SYBR Green I (SGI) is one of the fluorescent dyes which can specifically bind to double-stranded DNA chains or single-stranded DNA with G-quadruplex structure to produce strong fluorescence emission [39]. In addition, SGI possesses good optical physical properties, temperature stability, low fluorescence background in aqueous systems, excellent selectivity for specific DNA structures, and high sensitivity [39]. Therefore, it can be used in simple analysis of DNA conformation or utilized as fluorescent signal probe for aptamer biosensors [37,40]. Herein, a sensitive, simple and rapid method for quantitative analysis of tetracycline has been established based on aptamer-based, label-free fluorescence strategy using SGI as the signal probe. As illustated in Scheme 1, the tetracycline-binding aptamers in the natural state display the G-quadruplex spatial structures and can be recognized by SGI, resulting in a strong fluorescence emission. While with tetracycline present in the system, aptamers preferentially recognize and

bind to tetracycline, resulting in a change of the aptamer configuration, which is no longer recognized and inserted by SGI. As a result, the fluorescence of SGI declines. Thus, the quantitative analysis of tetracycline can be achieved by measuring the intensity of the fluorescent signal emitted by SGI.

2. Experimental

2.1. Reagents and materials

All chemicals used in the experiments were of analytical grade and used without any further purification or treatment. Tetracycline, oxytetracycline, and chloramphenicol were purchased from Bioengineering Co., Ltd. (Shanghai, China). Kanamycin, enrofloxacin, norfloxacin, and SYBR Green I (SGI) were bought from Sigma Aldrich (St. Louis, USA). The preparation and dilution of antibiotics solutions were completed with ultrapure water. Trichloroacetic acid and chloroform were obtained from Beijing Chemical Reagent Company (Beijing, China). The actual samples selected in the experiments are pure milk, acquired from local supermarket (Changchun, China).

The 76-mer ssDNA aptamers specific for tetracycline with the following sequence [30], 5'-CGT ACG GAA TTC GCT AGC CCC CCG GCA GGC CAC GGC TTG GGT TGG TCC CAC TGC GCG TGG ATC CGA GCT CCA CGT G-3', were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China). The aptamer solution was prepared with ultrapure water and stored at -18 °C before use.

2.2 Apparatus

The fluorescence spectra were measured on a RF-5301 fluorescence spectrophotometer (Shimadzu, Tokyo, Japan), with the slits of excitation and emission set at 5 nm. Ultrasonic operation was performed with a 125 KQ-300DE ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Shanghai, China). Vortex mixing was performed on a WH-3 vortex mixer (Huxi, Shanghai, China). The centrifugation was carried out on a CR20B2 refrigerated centrifuge (Tokyo, Japan). The circular dichroism (CD) spectra were acquired on a PMS 450 circular dichroism spectropolarimeter (BioLogic Science Instruments, France). CD spectra were recorded from 220 to 330 nm with the following parameters, scanning speed of 100 nm/min, bandwidth of 1 nm, response time of 5 s, and data interval of 0.2 nm. HPLC analysis of tetracycline was performed using a LC2010A HPLC system (Shimadzu, Tokyo, Japan) equipped with a C_{18} column (250 × 4.6 mm i.d., particle size 5 μm) and a UV detector.

2.3 General procedure for aptamer-based label-free fluorescence detection of tetracycline

In a typical experiment, in a 1.5 mL centrifuge tube, 50 μL different concentrations of tetracycline solution was added, vortex-mixed for 8 min with 50 μL 0.5 μM tetracycline-binding aptamers at room temperature. Then, 50 μL of 5 μg/mL SGI was added, vortex-mixed for 10 min and diluted to 500 μL with ultrapure water, with the final tetracycline concentrations of 1, 5, 10, 20, 30, 40, 50, 60 μg/mL. Subsequently, the fluorescence emission spectra were collected over the range from 510 nm to 650 nm with the excitation wavelength of 495 nm. Taking the SGI-aptamers solution without tetracycline as control, the fluorescence quenching efficiency $(F_0\text{-}F)/F_0$ was linearly proportional to the tetracycline concentration, in which F_0 and F equal to the maximum fluorescence emission of the ensembled solution in the absence and presence of tetracycline respectively. All experiments were performed at room temperature.

2.4 Pretreatment of milk samples

The procedure for the pretreatment of the actual samples (pure milk sold at a local supermarket) was carried out according to the published work [41] as follows. To start with, 2 mL of pure milk sample was added into a 10 mL centrifuge tube and diluted with 3 mL ultrapure water. Then, 1 mL 10% trichloroacetic acid and 1 mL chloroform were added and vortex-mixed for 1-2 min to precipitate proteins and dissolve other organic matters in the matrix. Subsequently, the mixture was ultrasonically treated at 20 ℃ for 15 min and centrifuged at 13,000 rpm for 10 min to separate the deposit. The supernatant was transferred into another centrifuge tube and centrifuged at 10,000 rpm for 10 min to remove the deposit once again and get the final supernatant used for fluorescent analysis.

2.5. HPLC detection of tetracycline in the actual samples

In order to verify the reliability of the proposed aptamer-based label-free fluorescent assay, the actual samples were also measured by HPLC according to Chinese National Standard GB/T 22990-2008 (Determination of oxytetracycline, tetracycline, chlortetracycline, doxycycline residues in milk and milk powder - HPLC-UV method). Firstly, 10 mL pure milk and 20 mL 0.1 M Na_2EDTA-Mcllvaine buffer solution were added into a 50 mL centrifuge tube, mixed under vortex for 2 min, then centrifuged at 10 ℃ and 5000 rpm for 10 min. The supernatant was transferred to another centrifuge tube. After that, added 20 mL buffer solution into the residue and repeated the extraction steps again. Then the two parts of supernatant were merged together for further purification. The supernatant solution was passed through the oasis HLB SPE column

which was activated with 5 mL methanol and 10 mL water before use. After elution with 5 mL methanol-water (1:19), the column was dried under reduced pressure for 5 min, and then eluted with 5 mL methanol. The collected eluent was passed through the carboxylic acid type cation exchange column, which was activated with 5 mL methanol before use, and then the column was washed with 5 mL methanol. After dried under reduced pressure, the column was eluted with 4 mL solution of 0.01 M oxalate-acetonitrile (1:1). The eluent was collected and dried under nitrogen blow at 45 °C. The residue was then dissolved in 2 mL of mobile phase solution and filtered through a 0.2 μm PVDF filter membrane to obtain the final HPLC sample. The operation parameters of HPLC were as follows: the mobile phase was 0.01 M oxalic acid/acetonitrile/methanol (77/18/5, v/v/v); the flow rate was 1.0 mL/min; the column temperature was maintained at 40 °C; the UV detection wavelength was set at 350 nm; the injection volume was 60 μL. The tetracycline standard solutions of 5, 10, 12.5, 17.5, 20, 25 μg/mL were prepared and tested by HPLC for the establishment of a standard curve for quantification.

3. Results and discussion

3.1 Design of fluorescent assay and detection principle

The aptamer-based, label-free fluorescence strategy was proposed for the detection of tetracycline, employing the intercalating dye SGI as the signal indicator, which showed an obvious fluorescence intensity when it is intercalated into the groove of dsDNA or distributed in the G-quadruplex structures [39]. The fluorescence spectra in Fig. 1 validated the feasibility of this assay protocol. SGI belongs to the asymmetrical cyanine dyes, and free SGI is nearly nonfluorescent in aqueous solution (Fig. 1, curve a). The fluorescence of SGI is very weak in the presence of tetracycline (Fig. 1, curve b), demonstrating that tetracycline itself does not influence the free state of SGI in the solution. However, the fluorescence of SGI can be dramatically enhanced upon binding to the aptamers (Fig. 1, curve c), with excitation and emission maxima at 495 and 530 nm respectively. Conversely, significant decrease of the SGI fluorescence was observed upon the association of aptamers with the target tetracycline (Fig. 1, curve d). The results indicated that, the aptamers itself has the configuration that enhances the fluorescence emission of SGI, whereas the specific and adaptive recognition of aptamers to the target tetracycline caused the conformational changes of aptamers, which cannot provide the microenvironment for SGI emitting fluorescence.

Nucleic acid aptamers fold into a flexible but well-defined three-dimensional structure upon

binding to their target molecules. Circular dichroism (CD) spectroscopy, an optical technique that measures the difference in the absorption of left and right circularly polarized light, is widely utilized to monitor the conformational changes of aptamers in different cases [42,43]. As illustrated by the CD spectra in Fig. 2, in the absence of tetracycline, there is a negative band at ~260 nm and a positive band at ~290 nm for the unbound tetracycline-binding aptamers (curve a), typically characteristic of antiparallel G-quadruplex structures [44]. G-quadruplex structures are four-stranded helical DNA structures, which are formed from guanine-rich sequences [45]. As a guanine-rich sequences (5'- CGT ACG GAA TTC GCT AGC CCC CCG GCA GGC CAC GGC TTG GGT TGG TCC CAC TGC GCG TGG ATC CGA GCT CCA CGT G-3'), the unbound tetracycline-binding aptamers mainly exist as G-quadruplex structures. On the other hand, the long-sequenced aptamers themselves will form some double helix structures. Thus, the SGI was inserted into the duplexes and G-quadruplex structures of aptamers, so exhibited strong fluorescence. However, upon specific interaction with tetracycline, a positive wavelength band at ~280 nm as well as a negative band at ~245 nm appear gradually (curve b), indicating that hairpin structure of aptamers came into being [46]. The specific interaction between an aptamer and its target is complemented through an induced fit mechanism, which requires the aptamer to adopt a unique folded structure to its target. Herein, the structure basis of this CD spectrum change can be attributed to the conformation change of aptamers from G-quadruplex structure into hairpin structure caused by the formation of tetracycline-aptamers complexes through supramolecular interactions. Consequently, from the CD spectra, it was confirmed that the specific binding of tetracycline to the aptamers can release SGI from the G-quadruplex aptamers, and correspondingly reduce the fluorescence emission of SGI, which laid the foundation of this label-free fluorescent strategy.

3.2 Optimization of experimental conditions

In order to improve the sensitivity of this assay, the experimental conditions for tetracycline detection were carefully optimized. As mentioned above, the proposed rapid analysis of tetracycline is based on the structural changes of aptamers themselves caused by binding to tetracycline, resulting in different effects of differently-structured aptamers on SGI fluorescence. Therefore, the assay performance will be influenced by factors such as the reaction medium, the intercalating time of SGI into aptamers, the incubation time of tetracyline with aptamers, the concentrations of aptamers and SGI.

First of all, it is particularly important to determine the reaction medium of this assay. The

feasibility analysis experiments were performed in ultrapure water, Tris-HCl buffer (50 mM, pH = 7.4) and PBS buffer (10 mM, pH = 7.4). The results demonstrated that the fluorescence emission intensity of SGI induced by aptamers in ultrapure water was much higher than that in Tris-HCl buffer and PBS buffer. Moreover, with the presence of tetracycline, the fluorescence emission of SGI decreased most significantly in ultrapure water, while the fluorescence decline caused by tetracycline in Tris-HCl buffer and PBS buffer was not noticeable. Accordingly, the most significant fluorescence quenching efficiency $(F_0\text{-}F)/F_0$ was observed in the reaction medium of ultrapure water (Fig. 3). So, ultrapure water was selected for investigation instead of normally used buffer solutions.

Since the proposed approach relies on the fluorescence change of SGI before and after the specific interaction between the target and aptamers, it is important to obtain a large fluorescence enhancement and high quantum yield of SGI upon binding to nucleic acid aptamers. Therefore, the kinetics of SGI intercalating into the tetracycline-binding aptamers was investigated, in order to explore the optimum intercalating time of SGI into aptamers with G-quadruplex structure. Fig.4 depicts the time course of SGI fluorescence signal in the sensing system after vortex mixing with aptamers for different time. The fluorescence signal gradually increases, and then levels off after 10 min. Therefore, the intercalating time of SGI into aptamers was performed at 10 min. Results indicate that the SGI is a very suitable dye for staining the G-quadruplex structure of tetracycline-binding aptamers, which forms the basis of this assay for tetracycline detection.

Subsequently, the effects of incubating time of tetracycline with aptamers on the fluorescence intensity were investigated. Briefly, tetracycline was vortex-mixed with aptamers for different time. Then SGI solution was added into the mixture, vortex-mixed thoroughly for 10 min and diluted to 500 μL with ultrapure water. The fluorescence spectra of the ensemble solutions were measured. As can be seen from Fig. 5, the fluorescence intensity decreases sharply within the first 6 min, then slows down, and remains constant after 8 min. Thus, 8 min was optimized as the incubation time of tetracycline with the aptamers.

Considering that the sensing strategy is based on the fluorescence change of SGI induced by the specific recognition of aptamers with tetracycline, the effect of SGI concentration on the performance of the assay was studied. The fluorescence responses were evaluated by measuring the fluorescence quenching efficiency $(F_0\text{-}F)/F_0$, where F_0 and F are the fluorescence intensity of the system in the absence and presence of tetracycline, respectively. As shown in Fig. 6, the highest fluorescence quenching efficiency was achieved with the SGI concentration at 0.5 μg/mL. Thus, the optimum SGI

concentration for this assay is chosen at 0.5 μg/mL.

Finally, appropriate amount of aptamers is critical to guarantee the feasibility of this assay. On the one hand, more aptamers provide more G-quadruplex structures for binding with SGI and causing fluorescence emission. On the other hand, higher concentration of aptamers would sacrifice the sensitivity of this assay. According to the results in Fig. 7, the fluorescence quenching efficiency of this assay protocol was maximized using 0.05 μM tetracycline-binding aptamers.

3.3 Analytical performance of target tetracycline detection

Under the above-mentioned optimal conditions, the sensitivity of fabricated label-free fluorescence aptasensor for tetracycline was investigated by analyzing different concentrations of tetracycline from 1 to 60 μg/mL. In a 1.5 mL centrifuge tube, 50 μL different concentrations of tetracycline standard solution was vortex-mixed with 50 μL tetracycline-binding aptamers (0.5 μM) for 8 min. Then 50 μL SGI (5 μg/mL) was added, vortex-mixed for 10 min and diluted to 500 μL with ultrapure water. The fluorescence measurements show that with the increasing amount of tetracycline, the fluorescence intensity of SGI was gradually quenched compared to that in the absence of tetracycline, which demonstrated that the binding between tetracycline and aptamers released the intercalated SGI from aptamers, in accord with the principle of this biosensing strategy. The fluorescence quenching efficiency of the system increased linearly versus the target concentration, indicating that tetracyline concentration can be quantitatively derived, and the detection limit (3σ) is calculated to be 0.2 μg/mL. Apparently, this method exhibits a comparable or even better sensitivity towards tetracycline compared to other reported colorimetric [31,47] and fluorescent [48] aptasensors, or chromatographic methods. In addition, this label-free fluorescent approach for tetracycline monitoring is very simple and cost-effective, only requiring vortex mixing to realize the assay, which can be completed within 20 min at room temperature.

Due to the fact that abuse of antibiotics will cause multiple antibiotic residues in food, other antibiotics may coexist with tetracycline and interfere with the detection, so the selectivity of the developed fluorescent method is of great significance to achieve the single target detection. The selectivity of this strategy for tetracycline was evaluated by challenging the sensing platform for other common antibiotics as contrast substances, including the structurally similar tetracyclines (oxytetracycline), the aminoglycosides (kanamycin), the fluoroquinolones (enrofloxacin, norfloxacin), and the amphenicols (chloramphenicol), at the same final concentration of 20 μg/mL. As illustrated in Fig. 8, only the interaction of aptamers with tetracycline (a) can cause significant changes in SGI

fluorescence, and the corresponding fluorescence quenching efficiency is nearly close to 100%, whereas the other five antibiotics (b-f) do not induce any remarkable fluorescence intensity changes. These results demonstrate that the proposed fluorescent strategy exhibits an adequate specificity towards tetracycline. The excellent selectivity is stemmed from the fact that aptamers can distinguish minor structural differences between targets and their analogs [36,49]. Previous studies on aptamers binding with tetracyclines revealed that the variation at 5-position or 6-position of tetracyclines determined the high levels of molecular discrimination of RNA or ssDNA aptamers against structural analogs [30,50,51].

3.4 Tetracycline detection in milk samples

To evaluate its applicability to real samples, the label-free fluorescent aptasensing strategy was applied for the analysis of tetracycline in milk samples. Although protein, fat and other organic substances have been removed during the procedure of sample pretreatment, some other special components in complex matrices might interfere with the aptamer-based assay using SGI as the fluorescent probe. Thus, as shown in Table 1, the effects of various potential interfering substances in milk (http://en.wikipedia.org/wiki/Milk#Nutritional_vaule) were assessed for the detection of 50 μg/mL tetracycline. The results demonstrate that no obvious interference was observed in the presence of these coexisting constituents for the detection of tetracycline. Thus, the established method herein could be applied to detect trace tetracycline in milk. The collected milk samples were pretreated according to the procedure described in Section 2.4. In the final matrix, different concentrations of tetracycline standard solutions were added and analyzed as requested in Section 2.3. With the increasing amount of tetracycline, the fluorescence intensity of the system was gradually decreased (Fig. 9). A plot of the fluorescence quenching efficiency versus the tetracycline concentration showed a linear calibration from 5 μg/mL to 25 μg/mL (inset in Fig. 9), and the detection limit was found to be 0.10 μg/mL. In order to validate the reliability of the proposed aptamer-based label-free fluorescent assay, three milk samples spiked with different concentrations of tetracycline were analyzed by this method and HPLC for comparison. As listed in Table 2, satisfactory recoveries from 98.98%-104.67% were obtained in spiked milk samples with the variation coefficient of 0.16-0.67%, and the results were consistent with those from HPLC. It can be seen that this method is stable and reliable for the quantitative analysis of tetracycline. In addition, including the sample pretreatment procedure, the whole analytical process could be completed within 1 h, demonstrating that this simple assay is suitable for the rapid screening of tetracycline in milk samples.

4. Conclusion

In summary, the present study elucidated the principle and feasibility of label-free fluorescent strategy for tetracycline detection using commercially available SYBR Green I (SGI) as the fluorescence signal reporter and tetracycline-binding aptamers as the recognition probe. As the fluorescent intercalator dye of nucleic acids, SGI has the ability to combine with tetracycline-binding aptamers with G-quadruplex structures and produce strong fluorescence emission. This assay relies on the turn-off mechanism of SGI fluorescence, based on target-induced conformational change of aptamers from G-quadruplex structures to hairpin structures. This fluorescent method was successfully applied to detect tetracycline in milk samples, with the detection limit as low as the national limited standard. Apparently, the proposed label-free assay eliminates the laborious and expensive process of DNA modification. The detection procedure is simple and convenient, which can be accomplished in homogeneous aqueous solution. After simple votex-mixing at room temperature, the change of SGI fluorescence will respond directly to the presence of tetracycline. Moreover, because of the high specificity between the aptamers and tetracycline, this aptamer-based fluorescent method exhibits excellent selectivity toward tetracycline versus other common antibiotics, so it would be expected to be used for the rapid screening of tetracycline in real samples.

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Figure captions

Scheme 1. The principle diagram about the label-free aptasensor for the detection of tetracycline based on the luminescence of SYBR Green I.

Fig. 1. Fluorescence spectra of SYBR Green I (SGI) in different systems. SGI, 0.2 μg/mL; aptamer, 0.1 μM; tetracycline (TET), 100 μg/mL.

Fig. 2. The CD spectra of tetracycline-binding aptamers (1.1 μM) before (a, black line) and after (b, red line) treated with tetracycline (0.5 μg/mL).

Fig. 3. Comparison of the fluorescence quenching efficiency（F_0-F）/F_0 of the system in different buffer. a, Pure water; b, Tris-HCl (50 mM, pH = 7.4); c, PBS (10 mM, pH = 7.4). SGI, 0. 2 μg/mL; aptamer, 0.1 μM; tetracycline, 20 μg/mL.

Fig. 4. Optimization of the reaction time between SGI and aptamers. Aptamer, 0.1 μM; SGI, 0.2 μg/mL.

Fig. 5. Optimization of the incubation time between tetracycline and aptamer. Aptamer, 0.1 μM; SGI, 0.2 μg/mL; tetracycline, 20 μg/mL.

Fig. 6. The fluorescence quenching efficiency of the system after addition of different concentrations of SGI. Aptamer, 0.1 μM; tetracycline, 20 μg/mL.

Fig. 7. Effects of the concentration of tetracycline-binding aptamers on the fluorescence quenching efficiency of the system. SGI, 0.5 μg/mL; tetracycline, 20 μg/mL.

Fig. 8. (A) Comparison of the fluorescence quenching efficiency (F_0-F)/F_0 of the system for different antibiotics: a, tetracycline; b, oxytetracycline; c, kanamycin; d, enrofloxacin; e, norfloxacin; f, chloramphenicol. SGI, 0.5 μg/mL; aptamer, 0.05 μM; antibiotics, 20 μg/mL. (B) Chemical structures of the tested antibiotics.

Fig. 9. Fluorescence spectra of the established aptasensor utilized for increasing concentrations of tetracycline in milk sample (5, 10, 12.5, 17.5, 20, 25 μg/mL). Inset is corresponding linear calibration plot of the fluorescence quenching efficiency (F_0-F)/F_0 versus tetracycline concentration. SGI, 0.5 μg/mL; aptamer, 0.05 μM.

Table 1. The influence of various potential interfering substances on the fluorescence intensity of

Interfering substances	Concentration in milk (g/L)	Added concentration (g/L)	Tolerance ratio	Change of fluorescence intensities (%)
K^+	1.36	1.36	1	9.7±5.5
Na^+	0.443	0.443	1	5.4±0.8
Mg^{2+}	0.103	10.3	10	8.6±1.4
Cl^-	1.07	1.07	1	17.5±0.1
NO^{3-}	0.158	0.158	1	1.2±0.4
Ca^{2+}	1.16	11.6	10	-2.4±1.4
Vitamin B_1	0.000474	0.00474	10	16.6±1.1
L-tryptophan	0.412	4.12	10	9.3±1.5
L-threonine	1.38	1.38	1	-3.7±0.9
Glycine	0.639	0.639	1	17.6±1.6
Glucose	0.186	1.86	100	2.9±0.4
L-lysine	2.72	2.72	1	19.0±0.6
Vitamin C	0.0237	0.237	50	-5.4±1.5
L-histidine	0.979	0.979	1	18.4±0.4
Lactose	52.0	104.0	2	2.5±1.4

the detecting system (*n*=3)

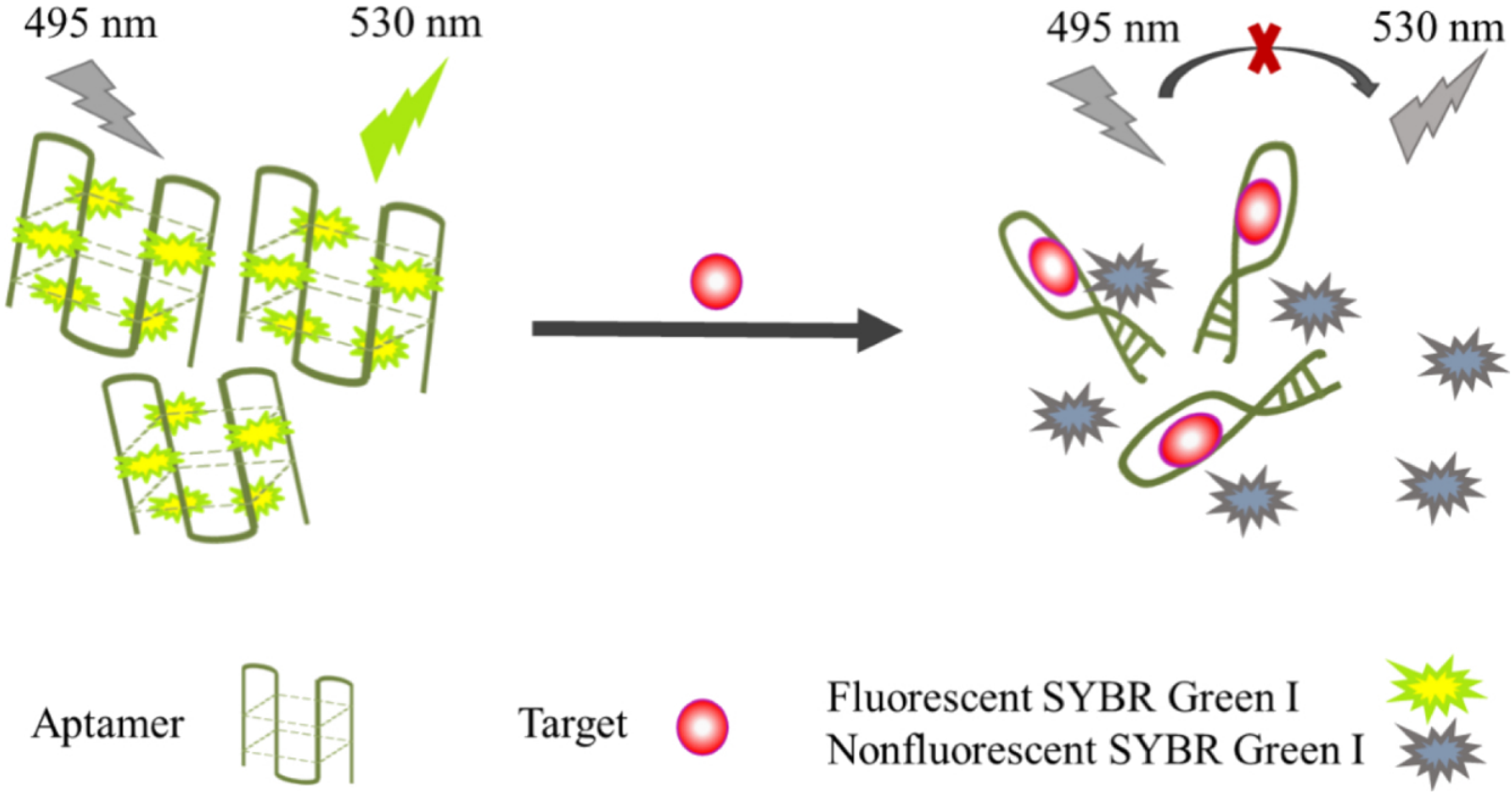
Notes: SG Ⅰ 0.5 μg/mL; aptamer 0.05 μM; tetracycline 50 μg/mL.

Table 2. Determination results for milk samples spiked with different amounts of tetracycline by this fluorescent method and HPLC (*n*=3).

Sample	Amount (μg/mL)	Amount found (μg/mL)		Recovery (%) ± RSD (%)	
		This method	HPLC	This method	HPLC
1	7.50	7.85	6.64	104.67 ± 0.16	88.48 ± 8.64
2	15.00	15.46	13.88	103.07 ± 0.67	92.50 ± 4.83
3	22.50	22.27	21.75	98.98 ± 0.43	96.66 ± 7.67

Highlights

1. Label-free aptamer-based fluorescent assay was established for tetracycline.
2. SGI as fluorescence signal probe can recognize conformational change of aptamers.
3. This assay needs no any covalent modification or chemical labeling.
4. This assay is simple, rapid, cost-effective and highly selective.



Graphics Abstract

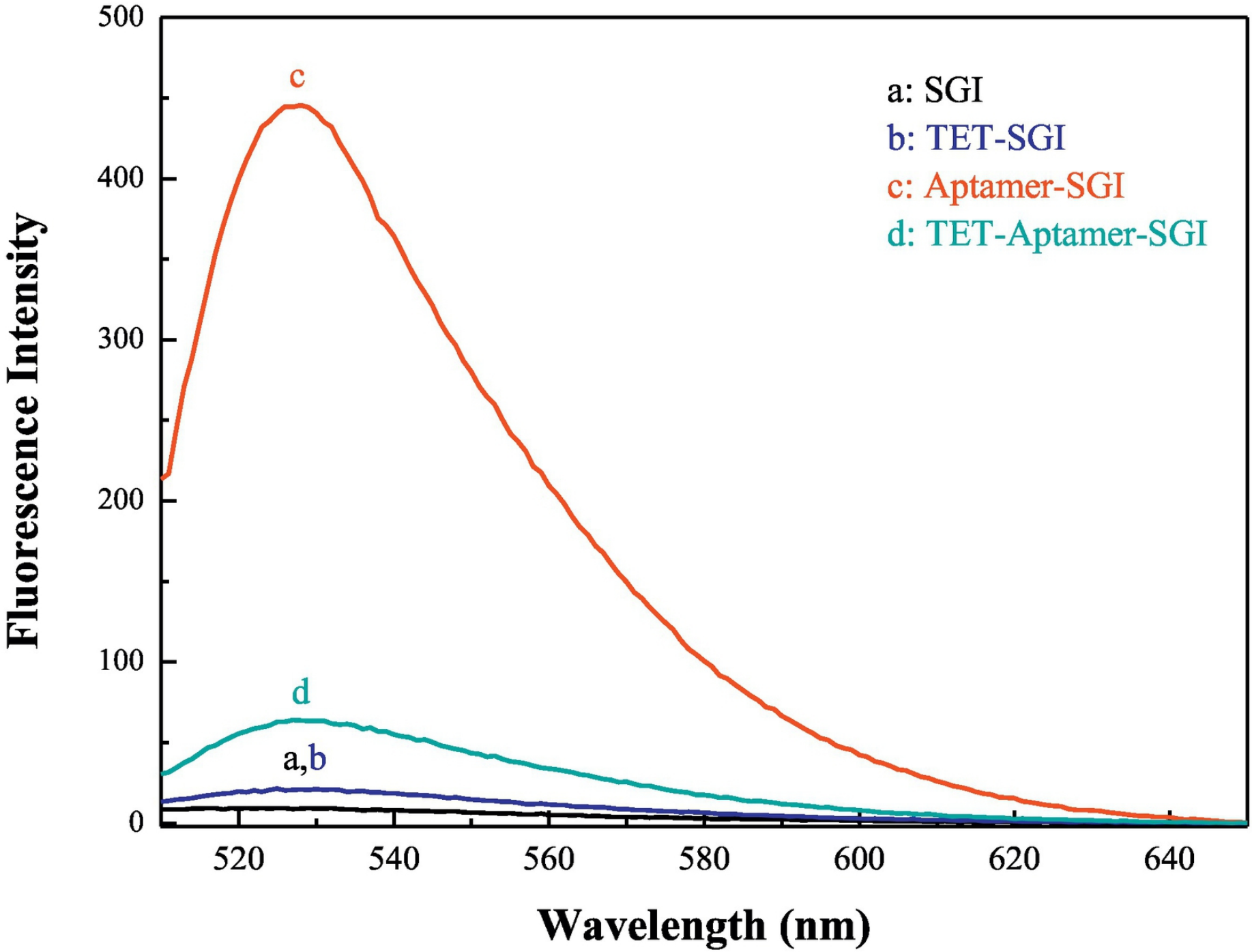

500
400
300
200
100
0
Fluorescence Intensity
520
540
560
580
600
620
640
Wavelength (nm)
a: SGI
b: TET-SGI
c: Aptamer-SGI
d: TET-Aptamer-SGI
c
d
a,b

Figure 1

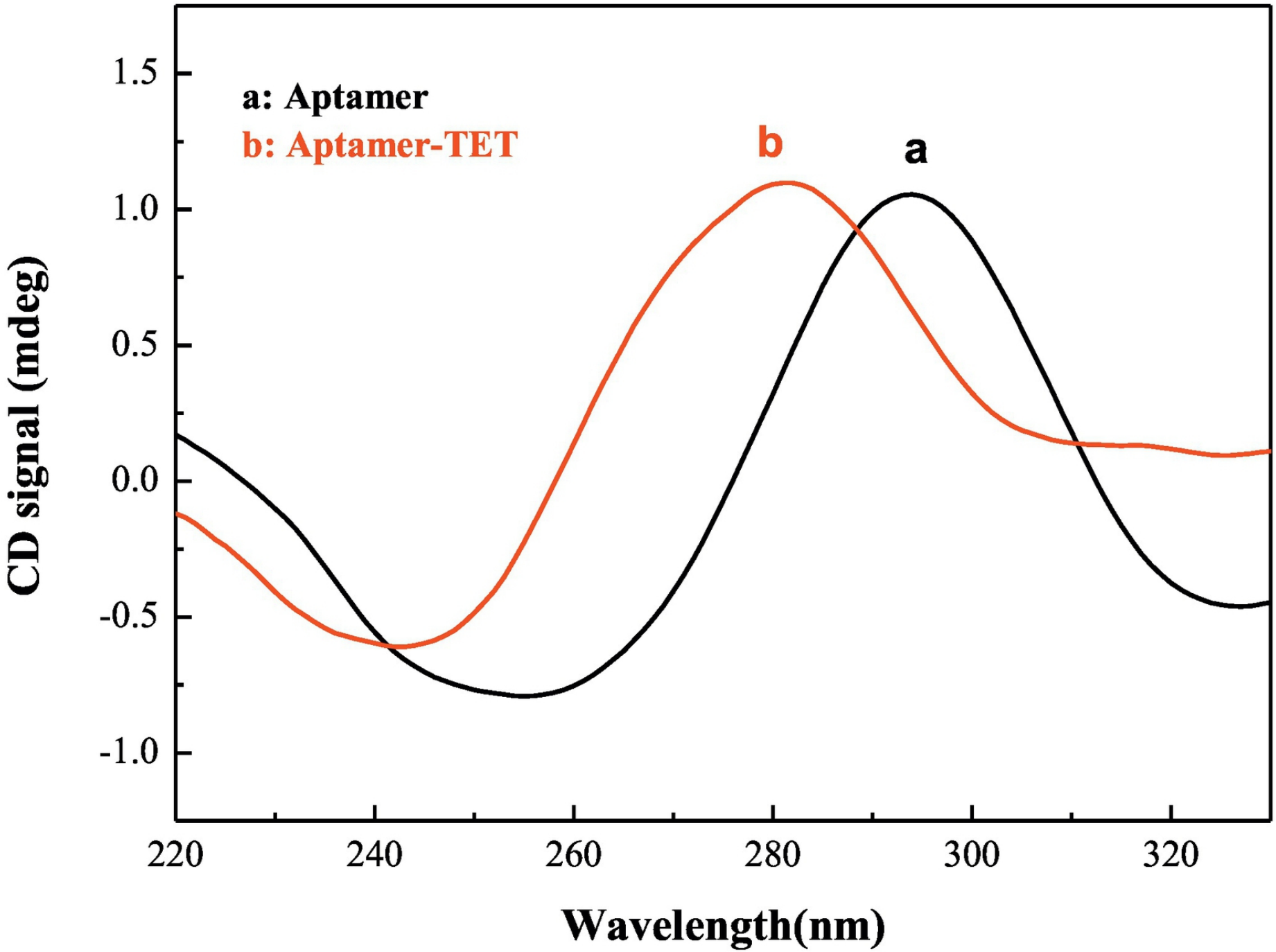

a: Aptamer
b: Aptamer-TET
b
a
1.5
1.0
0.5
0.0
-0.5
-1.0
CD signal (mdeg)
220
240
260
280
300
320
Wavelength(nm)

Figure 2

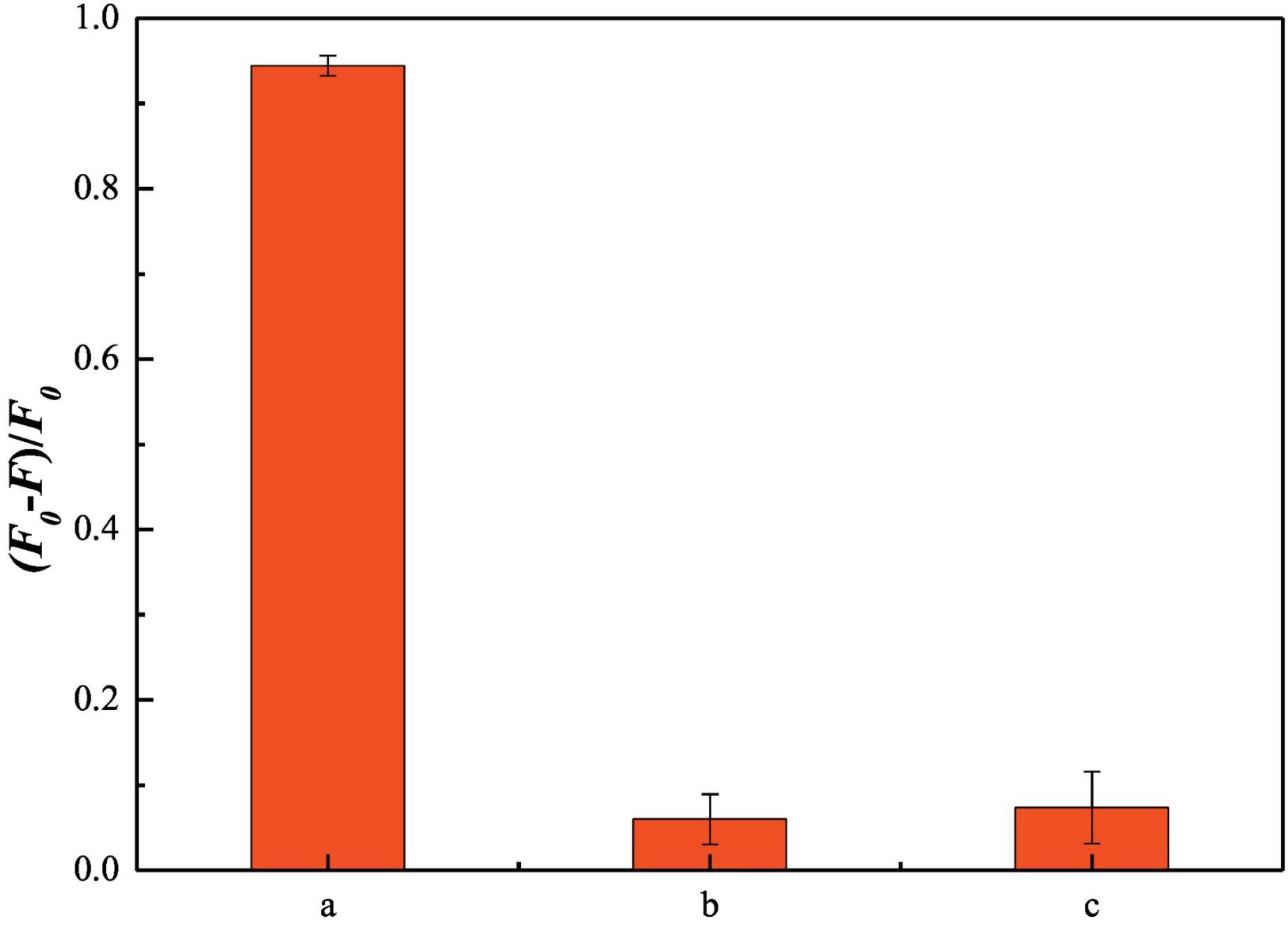


Figure 3

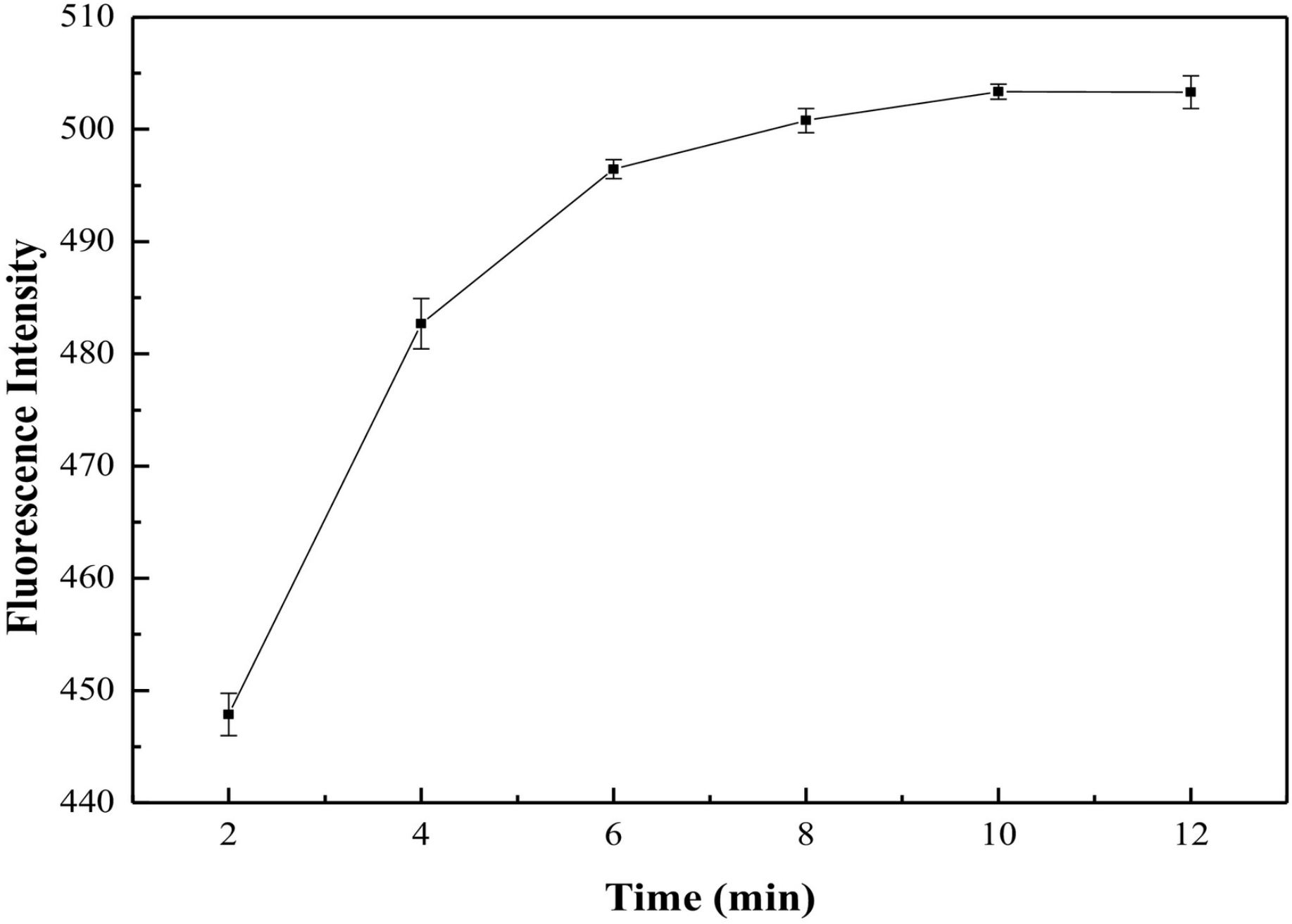

Fluorescence Intensity
510
500
490
480
470
460
450
440
2
4
6
8
10
12
Time (min)

Figure 4

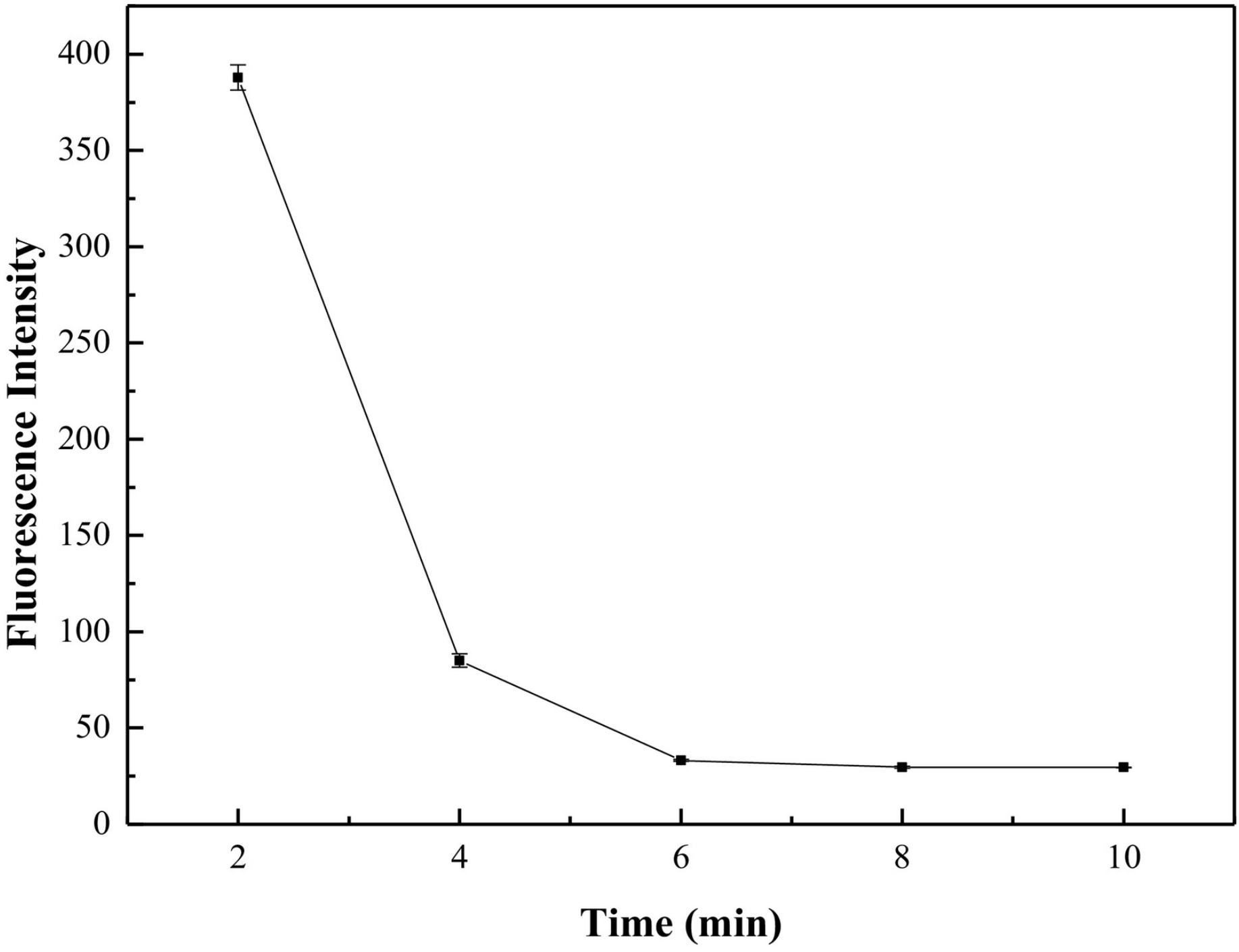

Fluorescence Intensity
400
350
300
250
200
150
100
50
0
2
4
6
8
10
Time (min)

Figure 5

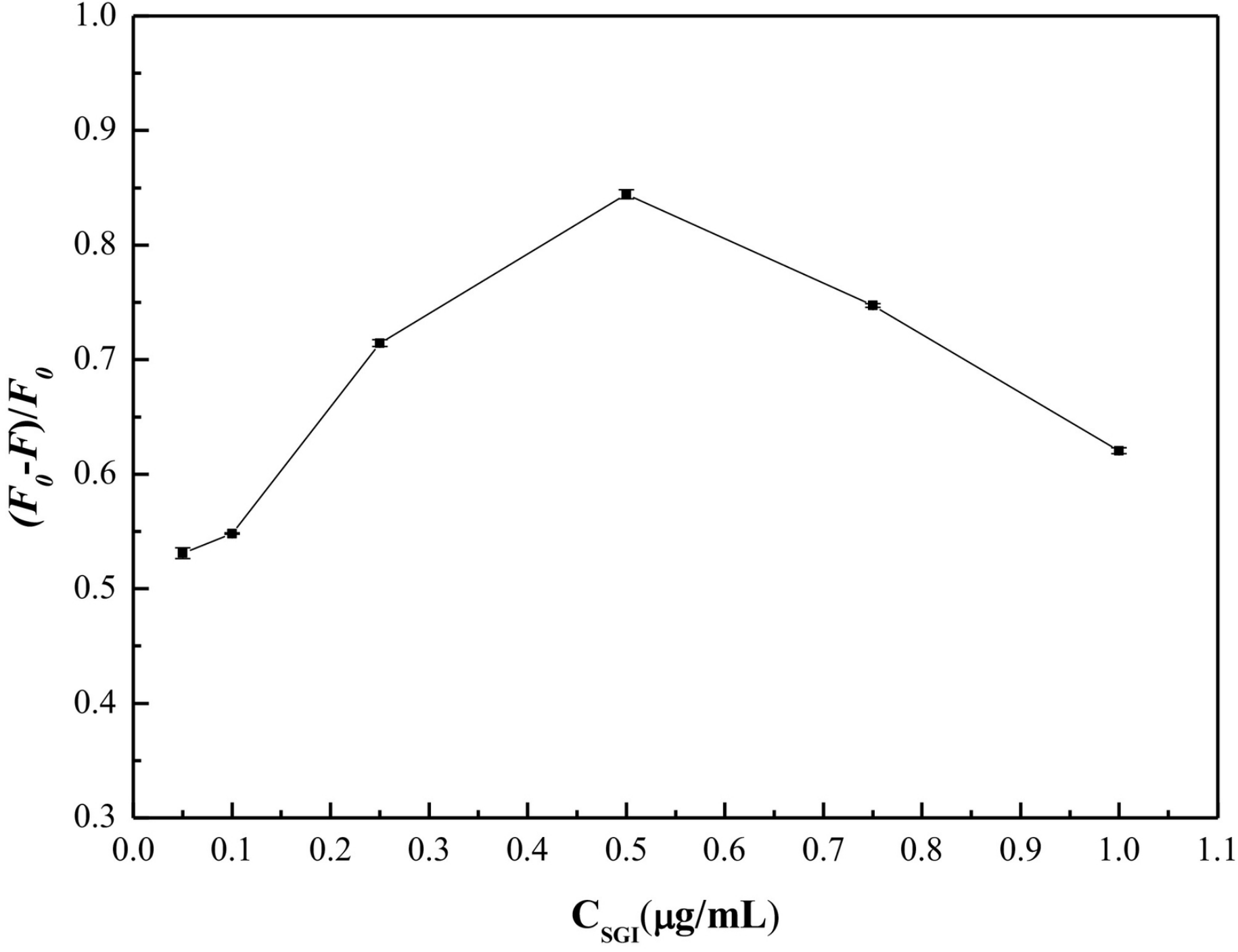

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
$(F_0-F)/F_0$
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
C_{SGI}(μg/mL)

Figure 6

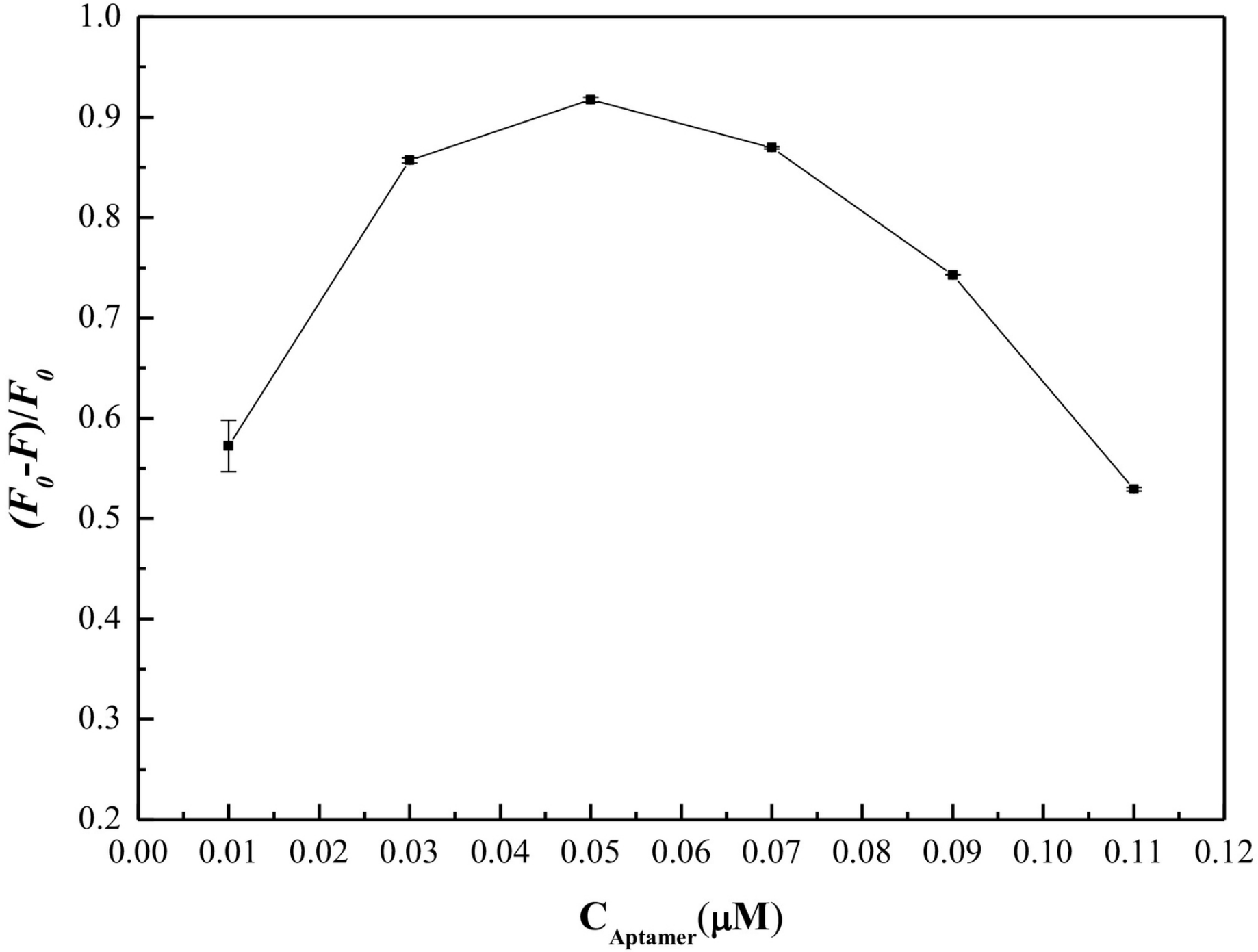
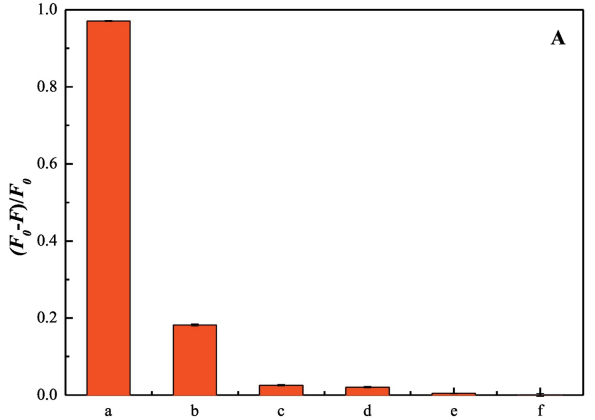

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
$(F_0-F)/F_0$
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0.11
0.12
$C_{Aptamer}(\mu M)$

Figure 7


A
1.0
0.8
0.6
0.4
0.2
0.0
$(F_0-F)/F_0$
a
b
c
d
e
f

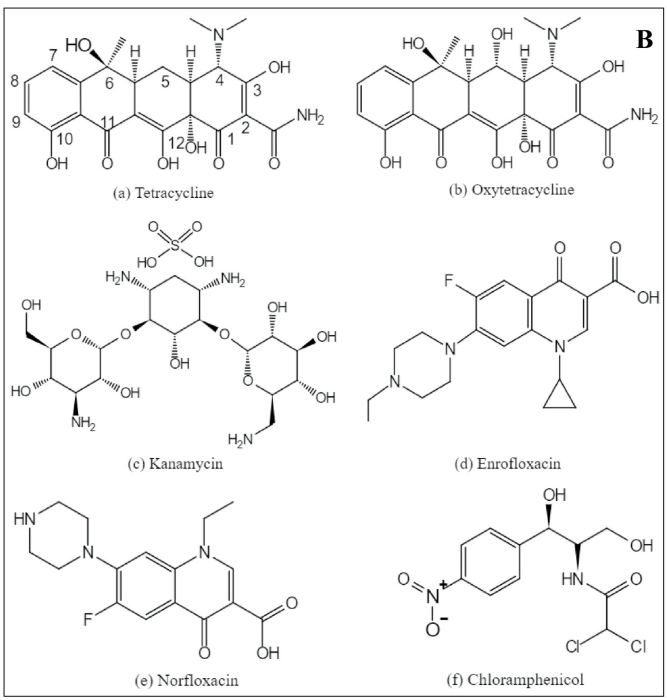

B
(a) Tetracycline
(b) Oxytetracycline
(c) Kanamycin
(d) Enrofloxacin
(e) Norfloxacin
(f) Chloramphenicol

Figure 8

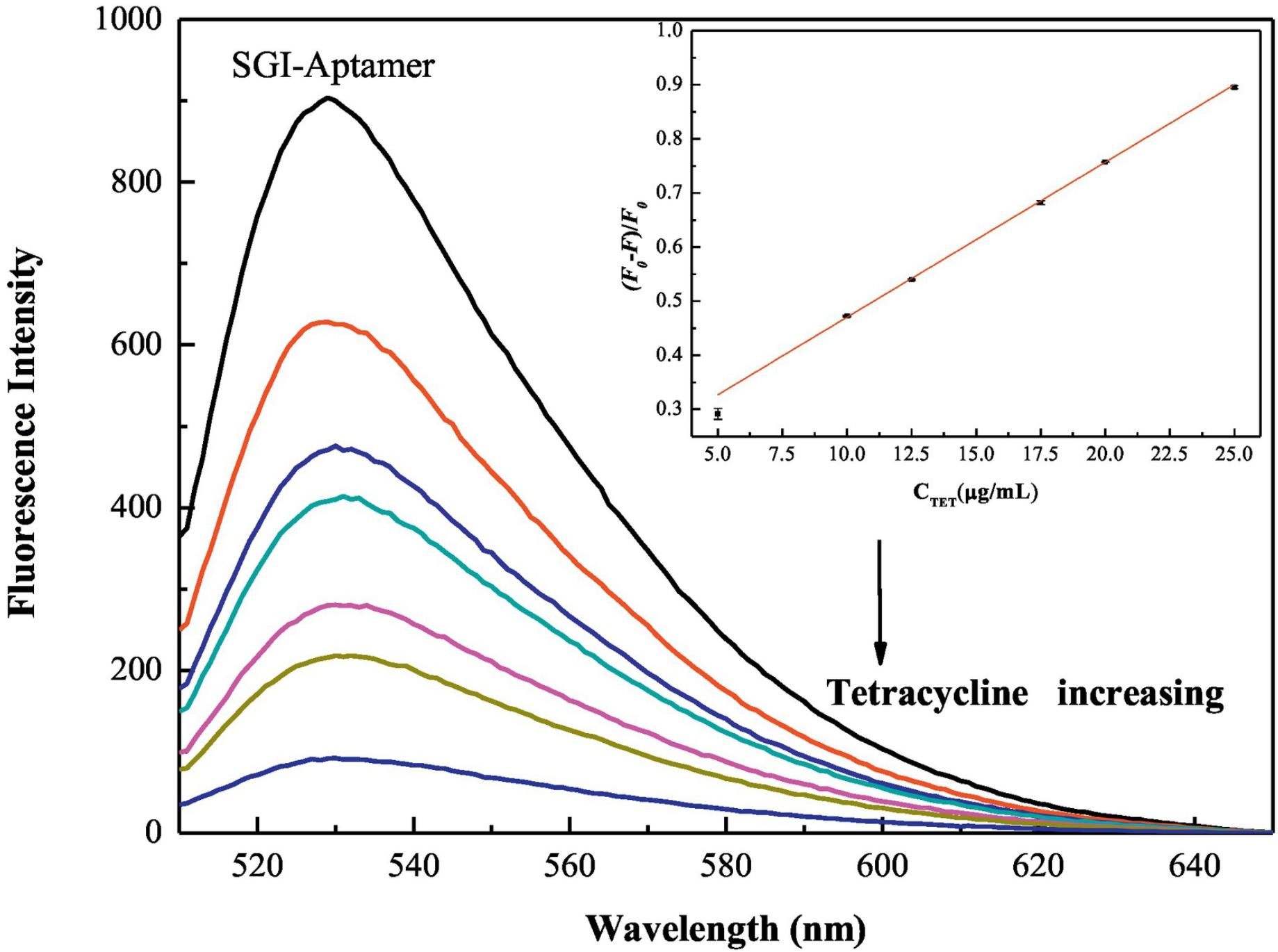

SGI-Aptamer
Fluorescence Intensity
Wavelength (nm)
Tetracycline increasing
$(F_0-F)/F_0$
C_{TET}(μg/mL)

Figure 9