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A simple FRET-based turn-on fluorescent aptasensor for 17β-estradiol determination in environmental water, urine and milk samples

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ABSTRACT

A competitive fluorescent aptasensor was constructed based on fluorescence resonance energy transfer (FRET) for determination of 17β-estradiol (E2). Initially, carboxyfluorescein (FAM)-labeled E2 aptamer and its partially complementary DNA modified with Black Hole Quencher 1 (BHQ1) formed aptamer/DNA duplexes and triggered fluorescence quenching. When E2 was added to compete for aptamer-binding sites with cDNA, FAM fluorescence was recovered as a result of cDNA release from the aptamer/DNA duplexes. With a competitive "turn-on" aptasensor, E2 could be quantitatively detected by monitoring fluorescence enhancement. Under optimized conditions, this assay exhibited a linear response to E2 within the range from 0.1 $ng \cdot mL^{-1}$ to 10 $\mu g \cdot mL^{-1}$, with the detection limit of 0.1 $ng \cdot mL^{-1}$ (0.35 nM). This assay has been successfully applied to determination of E2 in environmental, biological and food samples with low cost and simple pretreatment process, requiring no complicated operations. The results showed high selectivity and good recovery. The present strategy holds great potential in detection of E2 in complex matrixes and becomes important in environmental monitoring, food safety and human health.

1. Introduction

17β-estradiol (E2) is a type of steroidal estrogen that promotes animal growth and increases milk yield of milch cows, so that it has been widely applied in animal husbandry in recent years [1]. Many studies have reported that E2 is a typical environmental endocrine disrupting chemical (EDC) [2,3] and is contained in surface waters in diverse countries at the ng L^{-1} to mg L^{-1} levels [4,5]. Besides, some investigations have suggested that dairy waste contains up to 650 ng L^{-1} of endogenous estrogen E2 and other hormones [6]. Toxicological experiments have shown that even a low concentration of E2 residue entering organisms via the food chain could interfere with normal endocrine functions, resulting in detrimental effects, such as male infertility, blood-brain barrier damage, increased incidence of obesity and breast and testis cancers [7–9]. The use of E2 in animal husbandry has been banned in China. The National Environmental Protection Agency of the United States proposed in 2012 that the maximum residue of E2 in surface water was 1.47 pM, and Japan also enforced new regulations that E2 in drinking water was limited to 0.294 nM in 2015 [10]. Both the Codex Alimentarius Commission (CAC) and the National Food Safety Standards of China stipulated that estradiol could not be detected in animal foods. So it is of great significance to develop effective methods for sensitive monitoring of E2 in aqueous environmental, biological and food samples.

To date, instrumental analytical methods including high performance liquid chromatography (HPLC) [11] and gas chromatography-mass spectrometer (GC–MS) [12] have been extensively employed to detect E2 with high accuracy and sensitivity. However, these methods need complex sample pretreatment and professional technical skills. In addition, immunological assay methods based on antibodies, such as enzyme-linked immunosorbent assay (ELISA) [13], surface plasmon resonance (SPR) sensors [1] and chemiluminescence immunoassay [14], have showed high sensitivity and specificity in E2 detection. Nevertheless, antibody production is time-consuming and antibodies are susceptible to external environments including pH and temperature. Therefore, these methods are not suitable for simple and rapid monitoring of E2 residue in water, urine and food samples.

To address the above limitations, aptamers [15,16] were selected *in vitro* by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process from random-sequence nucleic acid libraries. They are

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single-stranded oligonucleotides (DNA or RNA) that bind to target molecules with high affinity and specificity. Aptamers have been developed as extremely promising substitutes for recognition elements of E2. So far, some assay methods based on aptamers have been published for E2 detection, such as colorimetric [17], electrochemical [18,19], photoelectrochemical [20] and spectroelectrochemical methods [21]. Colorimetry based on AuNPs has the advantages of low cost and simple operations, but it is susceptible to sample matrixes so that water samples in complex environments must be diluted and purified. Even though electrochemical or photoelectrochemical aptasensors always show high sensitivity and selectivity, their stability and reproducibility are still a major challenge, and water and urine samples need further extraction or dilution before E2 detection [22]. Moreover, the synthesis process of nanomaterials used for modifying electrodes is complicated and time-consuming. Aptamer usually needs to be modified and immobilized on the electrodes in a complicated immobilization process. Therefore, it is crucial to develop an aptamer-based method for detecting E2 in both water and urine samples without dilution or extraction or E2 in milk samples with a simple dilution process.

In this study, we successfully developed a novel "turn-on" aptasensor based on fluorescence resonance energy transfer (FRET) by using carboxyfluorescein (FAM) and Black Hole Quencher 1 (BHQ1) as the donor and acceptor pairs. In the absence of the target, fluorescein-labeled E2 aptamer naturally binds to its partially complementary DNA strand (denoted as cDNA) modified with a quencher. And then, fluorescent signals were highly efficiently quenched due to close proximity of the quencher to the fluorophore. Upon E2 addition, the aptamer preferred to form aptamer/E2 complexes rather than aptamer/cDNA duplexes. This was because cDNA was released from the fluorescein-labeled aptamer, resulting in FRET disruption and fluorescence increase. The test results showed that the fluorescence intensity was proportional to the E2 concentration. This aptasensor has been successfully utilized for determination of E2 in environmental, biological and food samples. Even though the competitive FRET-based aptasensor has been reported previously, it has never been used for the detection of E2, especially in real samples.

2. Materials and methods

2.1. Reagents and apparatus

17β-estradiol (E2), estriol (E3), progesterone (P4), dienestrol (DS), bisphenol A (BPA), tetrabromobisphenol A (TBBPA), 19-nortestosterone (19-NT) and tris(hydroxymethyl)aminomethane (Tris) (purity > 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was purified by a Milli-Q system (Millipore, Bedford, MA, USA) with resistivity of 18.2 MΩ cm. All other reagents (analytical grade) were purchased from Beijing Chemical Reagent Company (Beijing, China) and used without further purification or treatment. Tris – HCl buffer (100 mM Tris – HCl, 200 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$ and 5% ethanol; pH 8.0) was used for fluorescent assay.

Fluorescence intensities were recorded with a multifunctional microplate reader of Tecan Infinite 200 (Tecan Austria GmbH, Austria) with the excitation and emission lengths of 488 nm and 520 nm, respectively.

Modified oligonucleotides were chemically synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) and purified by HPLC. The sequence of fluorescein-labeled E2 aptamer [18] was 5′-GCT-TCC-AGC-TTA-TTG-AAT-TAC-ACG-CAG-AGG-GTA-GCG-GCT-CTG-CGC-ATT-CAA-TTG-CTG-CGC-GCT-GAA-GCG-CGG-AAG-C-$(CH_2)_6$-FAM-3′. The sequence of partially complementary DNA (cDNA) was 5′-BHQ1-$(CH_2)_6$-GCT-TCC-GCG-CTT-CAG-CGC-GCA-GCA-A-3′.

2.2. Aptamer-based fluorescent assay for E2 detection

E2 aptamer and cDNA were dissolved and diluted with Tris – HCl buffer. Then, 50 μL of E2 aptamer solution (0.1 μM) was mixed with 50 μL of cDNA solution (0.2 μM). The mixture was incubated at room temperature for 1 h in a brown centrifuge tube. Then, 150 μL standard solution of E2 was added at different concentrations. After they were incubated at room temperature for 2 h, the fluorescence intensity at the excitation/emission wavelength of 488/520 nm was recorded by a multifunctional microplate reader of Tecan Infinite 200 (Tecan Austria GmbH, Austria). Each concentration was measured for five times.

2.3. Preparation of real samples

Tap water samples were collected from our laboratory. Environmental water samples were collected from the Black Bamboo Park (Haidian, Beijing, China). Urine samples were offered by a female volunteer. The water and urine samples were filtrated through 0.22 μm membrane to remove particles. Milk samples used in this work were obtained from a local supermarket. After 5 mL of milk sample was centrifuged at 1000 × g for 10 min at 4 °C and diluted with Tris buffer (1:10, v:v), the upper fat layer was discarded. All four kinds of sample were tested with this method before spiking. To prepare spiked samples, a 1 μg mL^{-1} 17β-estradiol stock solution was prepared firstly and then was added to four kinds of samples to the final concentrations (1, 10 and 100 ng mL^{-1}) before performing the pretreatment. In other words, all the above mentioned samples were spiked with E2 stock solution at a series of concentrations of 1, 10, and 100 ng mL^{-1}. All samples were then stored at 4 °C.

3. Results and discussion

3.1. Principle of the "turn-on" fluorescent biosensor

In this study, a simple strategy was developed using a "turn-on" aptasensor based on FRET. As shown in Fig. 1, E2 aptamer was labeled with FAM at the 3′-end and its partially complementary DNA (cDNA) was modified with BHQ1 at the 5′-end. When aptamer and cDNA formed a duplex structure, only a low level of fluorescence was produced in the system because the fluorophore and quencher were situated in close proximity. In the presence of E2, a structural switch was induced by target binding for the aptamer, resulting in the release of cDNA and recovery of the fluorescence intensity. The fluorescence intensity increase is directly proportional to the concentration of E2.

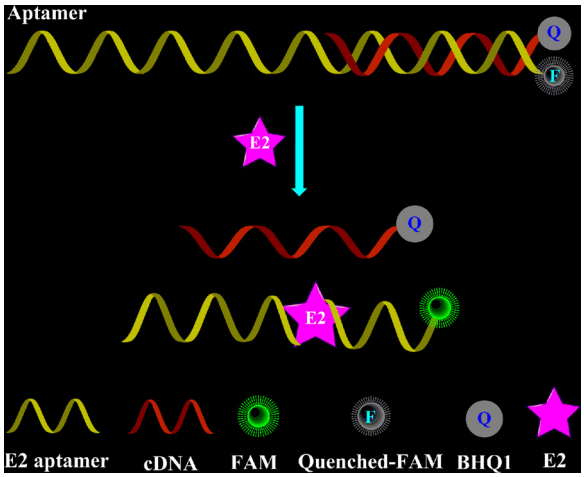

Fig. 1. Schematic illustration of the proposed fluorescent aptasensor for E2 detection. F = fluorophore (FAM) and Q = quencher (BHQ1).

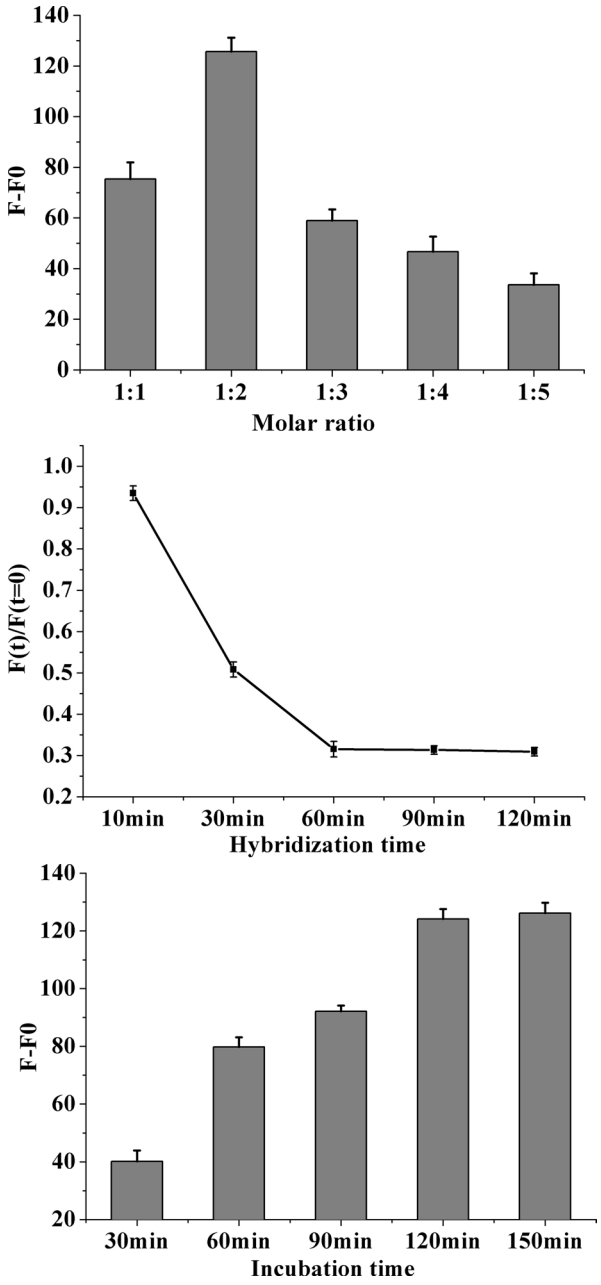

Fig. 2. Optimization of the concentration of cDNA, hybridization time and incubation time. (a) The fluorescence signal difference (F - F0) was used in response to 100 ng mL-1 E2 to determine the molar ratio of aptamer to cDNA. (b) E2 aptamer solution (0.1 μM) was mixed with cDNA solution (0.2 μM) in a brown centrifuge tube and incubated at room temperature. F(t)/F(t = 0) presented the ratio of the fluorescence intensity with certain incubation time (F(t)) to that without incubation (F(t = 0)). (c) The target detection was carried out to optimize the incubation time according to the procedure described in the "Materials and methods" section. The concentration of E2 was 100 ng mL-1. All error bars were obtained from three parallel experiments.

3.2. Optimization of reaction conditions

To achieve high sensitivity for E2 detection, it is necessary to optimize key parameters such as the cDNA concentration, time for hybridization of aptamer with cDNA and incubation time for the aptasensor to interact with E2.

Using E2 aptamer at a fixed concentration of 0.1 μM, the molar ratio of the aptamer to cDNA was investigated in order to achieve the optimal sensing performance. As shown in Fig. 2a, in the presence of 100 ng mL^{-1} E2, fluorescence signal enhancement reached the maximum fluorescence difference (F - F0, F represented the fluorescence signal obtained in the presence of the target; F0 represented that obtained without the target) when the concentration of cDNA was 0.2 μM. The result showed that the assay sensitivity deteriorated even though a higher concentration of cDNA could provide lower background fluorescence by forming stable aptamer/cDNA duplexes. We presumed that deficient amounts of cDNA would result in unstable or a small number of aptamer-cDNA duplexes and maintain high fluorescence in the experimental system. On the contrary, too much cDNA would affect the interaction between E2 and its aptamer and even cause nonspecific quenching, which would restrict the increase of fluorescence and lead to weak fluorescence signals. Therefore, 1:2 was the most optimal molar ratio of aptamer to cDNA for E2 detection.

To optimize the sensing performance, the time for hybridization of aptamer with cDNA was also evaluated. As shown in Fig. 2b, the fluorescence intensity decreased dramatically within 60 min and achieved the maximum quenching, suggesting that 60 min was adequate for the formation of aptamer/cDNA duplexes. Therefore, the optimal hybridization time was determined as 60 min for further sensing experiments. The hybridization time of 1 h is required because the aptamer fragment is relatively long, and it takes a certain amount of time for the aptamer to open its own conformation at room temperature and then hybridize with the complementary strand.

Finally, in order to obtain a desirable fluorescence response, the incubation time for the target to bind to aptamer was investigated. Since estradiol competes with the complementary strand for binding to the aptamer, the length and binding position of the complementary probe would affect the assay time for competitive binding. If the complementary strand is too long, a more stable secondary structure for hybridization between the aptamer and the complementary strand indicates a longer time for double strand opening after the addition of estradiol. In addition, if the fragment of the complementary aptamer is not an effective site for binding of the aptamer to estradiol, the fluorescence signal may not be restored or the recovery rate is low after the addition of estradiol, so that the fluorescence aptasensor cannot be established. In contrast, if the position of the complementary aptamer is the key site for the binding of the aptamer to estradiol, the recovery of the fluorescence signal is sensitive after the addition of estradiol. According to the relevant literature [18], the 25-mer complementary probe was used in this study. After the addition of estradiol, the combination of the aptamer and estradiol opened the double-stranded structure, so that fluorescence could be restored to the maximum after binding for enough time. As shown in Fig. 2c, the fluorescence intensity increased gradually and reached a plateau at the binding time of over 120 min. Therefore, in the following experiments, all fluorescence detection was initiated two hours after the addition of the target. Although the detection time was longer than colorimetry [23], it was far less than electrochemical aptasensors [24] and other biosensors [13].

3.3. Sensitivity of the aptasensor

Under optimal conditions, the aptasensor was used for quantitative determination at a series of E2 concentrations (0 ng mL^{-1} to 10 μg mL^{-1}) with the excitation and emission wavelengths of 488 nm and 520 nm, respectively. As shown in Fig. 3, the fluorescence intensity was enhanced with increased E2 concentrations in a wide linear range from 0.1 ng mL^{-1} to 10 μg mL^{-1}. The calibration curve of the fluorescence intensity was linearly related to the logarithm of the E2 concentration (R^2 = 0.985), with a detection limit of 0.1 ng mL^{-1} (0.35 nM). The sensitivity of the present method was higher than the previous reports [1,4,25–27], indicating that the "turn-on" fluorescent aptasensor effectively improved the sensitivity and could be used for E2 determination in real samples.

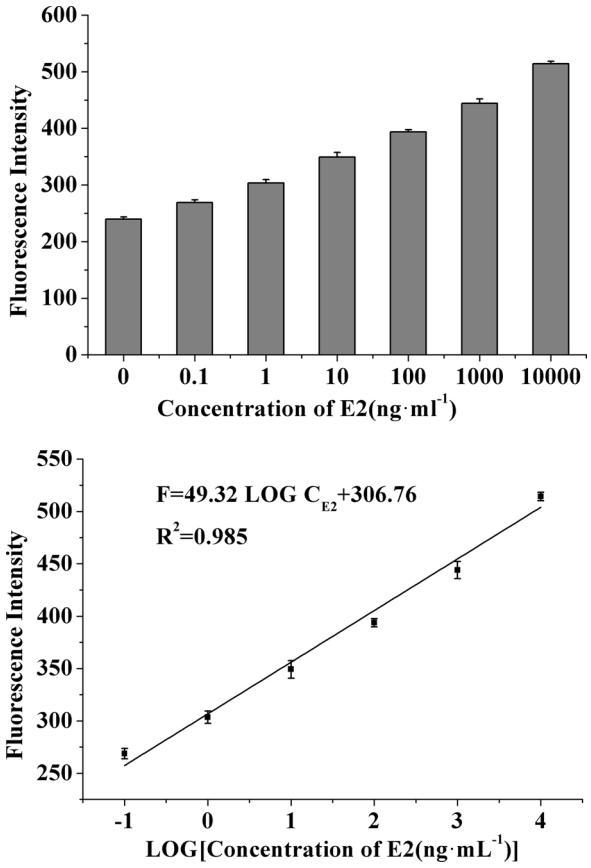

Fig. 3. (a) Fluorescence intensities upon addition of E2 at various concentrations (n = 3). (b) Linear relationship between the fluorescence intensity and logarithm of the E2 concentration (n = 3).

3.4. Selectivity of the aptasensor

Complicated sample matrixes may affect E2 analysis using the aptasensor. So the selectivity of the assay is an important issue for practical E2 detection. In order to determine the specificity of this method, other endocrine disrupting chemicals (E3, P4, DS, BPA, TBBPA and 19-NT) of 1 μg mL^{-1} were measured under the same experimental conditions as E2. As shown in Fig. 4, only E2 and the mixture (containing E2) induced a significant increase in the fluorescent intensity. It turned out

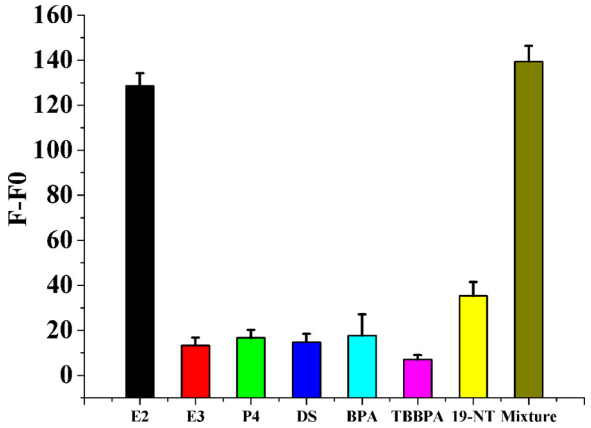

Fig. 4. Selectivity of the aptasensor towards E2 (100 ng mL-1) and other endocrine disrupting chemicals (1 μg/mL) (n = 3). F represented the fluorescence intensity in the presence of E2 or other endocrine disrupting chemicals (E3, P4, DS, BPA, TBBPA and 19-NT) and F0 represented the fluorescence intensity in the absence of any endocrine disrupting chemicals.

Table 1
Determination of 17β-estradiol (E2) in actual samples (n = 5).

Samples	Found (ng mL^{-1})	Spiked (ng mL^{-1})	Determined (ng mL^{-1})[a]	Recovery (%)	CV (%)[b]
Tap water	–	1	0.98	96.3–103.0%	3.8%
		10	10.12	98.0–103.4%	2.8%
		100	101.69	98.2–104%	3.1%
Environmental water	–	1	0.99	96.8–103.7%	3.9%
		10	10.10	96.2–106.9%	5.4%
		100	103.58	99.1–106.6%	4.0%
Urine	0.15	1	1.14	96.3–105.9%	5.4%
		10	10.25	91.4–107.5%	8.5%
		100	102.61	92.9–110.8%	9.0%
Milk	–	1	1.05	93.5–111.7%	10.3%
		10	10.50	92.4–120.6%	14.3%
		100	103.82	93.4–117%	12.1%

-: Not detected.
[a] Mean of three measurements.
[b] Coefficient of variation.

that the developed aptasensor had good specificity towards E2.

3.5. Practical applications of the aptasensor

To evaluate the applicability and reliability of this method, the assay was utilized to detect E2 in spiked environmental water, tap water, urine and milk samples. All samples were selected and spiked with E2 at 1, 10 and 100 ng mL^{-1}, respectively. Five repeats were measured for each sample to determine the accuracy of the assay. As shown in Table 1, the results were satisfactory for these four kinds of real samples. The average recoveries were in the ranges of 96.3–104.0%, 96.2–106.9%, 91.4–110.8% and 92.4–120.6% for environmental water, tap water, urine and milk samples, respectively. Their coefficients of variation (CVs) were 2.8–3.8%, 3.9–5.4%, 5.4–9.0% and 10.3–14.3%, respectively. These results demonstrated that the assay developed in this study could be useful as a quantitative method for E2 detection in real complex samples. It was worth noting that a complex multi-step pretreatment procedure was required for detecting E2 by other biosensors or HPLC methods, while only the simple pre-filtration (and additional pre-dilution for milk samples) process sufficed for this aptasensor. Among the detection methods reported (Table 2), the current method possessed lower sensitivity than some electrochemical assays but had many merits. For example, this aptasensor showed high tolerance to complex matrix interference even without complicated sample pretreatment. Besides, the fabrication of the sensor did not require a complicated aptamer immobilization process, professional and time-consuming nanomaterial synthesis and modification of electrodes. In summary, fluorescence aptasensors could achieve E2 detection with high sensitivity, high selectivity, simple pretreatment and easy operations.

4. Conclusions

In this work, a new "turn-on" fluorescent aptasensor with high selectivity and excellent recovery has been successfully developed for E2 detection in complex samples. It is worth mentioning that the method was carried out following one-step pre-filtration for detection of complex water and urine samples and two-step pretreatment (centrifugation and dilution) for detection of milk samples. Moreover, the aptasensor was successfully used for E2 determination among environmental, biological and food samples. Even though the competitive FRET-based aptasensor has already been reported, the method has never been used for the detection of E2, especially in real samples. In general, the aptasensor developed in this study is a promising alternative for the current methods and is important for E2 detection in environmental monitoring, food safety and human health.

Table 2
Comparison between the present work and other reported techniques for E2 determination.

Analytical techniques	Sensors fabricated	Detection limit (nM)	Linear range (nM)	Demonstration in samples			Reference
				Biological	Environmental	Food	
Fluorescence	Aptamer + 17β-estradiol-BSA + Evanescent wave all-fiber	2.1	–	–	√	–	[4]
	Aptamer + Gold nanoparticles + Fluorochrome(Rhodamine B) + NaCl	0.22	0.82–20.5	–	√	–	[28]
	Aptamer + Ru complex + quantum dots	37	80–400	√	–	–	[26]
Colorimetry	Aptamer + Gold nanoparticles + NaCl	0.35	$0.35–3.5 \times 10^4$	–	–	–	[17]
Electrochemical voltammetry	MIP + MNA + PtNPs/GCE, Aptamer immobilization	16	$30–5 \times 10^4$	–	√	–	[27]
Electrochemical impedance spectra	Nanoporous conducting polymer (Py-co-PAA) + Ag/AgCl/GCE, Aptamer immobilization	1×10^{-6}	1×10^{-6}–1×10^3	√	√	–	[29]
Electrochemiluminescence	Gold electrode + cDNA + f $Ru(bpy)_3^{2+}$, Aptamer immobilization	1.1×10^{-3}	0.01-10	√	√	–	[24]
Surface-enhanced Raman spectroscopy	Aptamer + Au @ Ag CS NPs + 4-MBA	5×10^{-5}	1×10^{-4}–10	–	√	–	[10]
Transistor device output current	Carbon nanotube field effect transistors (CNT FETs), Aptamer immobilization	50	$50–1.6 \times 10^3$	–	–	–	[30]
Nanoparticle size	Carboxylated nanoparticles (NPs), Aptamer immobilization	5	5–100	–	–	–	[31]
Fluorescence	FAM-labeled aptamer + BHQ1 modified cDNA	0.35	$0.35–3.5 \times 10^4$	√	√	√	This work

BSA: bovine serum albumin; MIP: molecular imprinting polymer; MNA: 6-mercaptonicotinic acid; PtNPs/GCE: platinum nanoparticle-modified glassy carbon electrode; 4-MBA: 4-mercaptobenzoic acid.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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