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A simple fluorescence anisotropy assay for detection of bisphenol A using fluorescently labeled aptamer

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ABSTRACT

Bisphenol A (BPA) is one of the environmental endocrine disruptors (EDCs), and BPA contamination in environment can cause high risks to human health. Rapid determination of BPA on sites is in high demand in environmental analysis. Taking advantage of aptamers as affinity ligands and fluorescence anisotropy (FA) analysis, we developed a simple and rapid FA assay for BPA by employing a single tetramethylrhodamine (TMR) labeled short 35-mer DNA aptamer against BPA. The assay is based on the BPA-binding induced conformation change of TMR-labeled aptamer and alteration of interaction between TMR and guanine bases, resulting in change of FA signals. We screened the FA change of aptamer probes having TMR label on a specific site of the aptamer upon BPA addition. The aptamer with a TMR label on the 22nd T base showed large FA-decreasing response to BPA and maintained good binding affinity to BPA. By using this TMR-labeled aptamer, we achieved FA detection of BPA with a detection limit of 0.5 μmol/L under the optimized conditions. This assay was selective towards BPA and enabled the detection of BPA spiked in tap water sample, showing the potential applications on water samples.

Introduction

Bisphenol A (BPA), one of typical endocrine disrupting chemicals (EDCs), has attracted great concerns as its structure is similar to natural hormones (Krishnan et al., 1993). It disturbs the functions of the hormone estrogen through binding to estrogen reporters, leading to endocrine disorders. BPA can affect sex hormones functions, reproductive organs, thyroid functions, and immune system (Maffini et al., 2006; Sheng et al., 2019; Siracusa et al. 2018; Tao et al., 2016). Some new toxicology studies showed that BPA exposure is also associated with obesity, diabetes, cardiovascular diseases, and behavioral problems (Diamanti-Kandarakis et al., 2009; Ma et al., 2019; Michalowicz, 2014; Vom Saal et al., 2012; Yin et al., 2015). BPA is widely used for the manufacture of polycarbonate and epoxy resins based products, such as food packing, toys, tableware, and etc. It easily migrates into food, surface water and soil from packing materials, wastewater and landfill (Ashfaq et al., 2018; Kang et al., 2006; Rezg et al. 2014; Rochester, 2013; Rubin, 2011). Considering the adverse effects and continuous leaching of BPA, the determination of BPA is of great significance on environmental monitoring and human health. Traditional methods for BPA analysis are chromatographic methods (Ballesteros-Gomez at al., 2009), which have advantages of high sensitivity, good selectivity, and accuracy. While the disadvantages including the need for complicated sample preparation, time-consuming operation and

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professional operators in the laboratory may limit their use in rapid analysis on sites. Some antibody based sensing platforms have been reported for BPA detection, but they may suffer drawbacks in high cost and poor stability of antibodies (Marchesini et al., 2005).

As alternatives of antibodies, aptamers are artificially synthesized single stranded DNA or RNA by in vitro systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Aptamers show some advantages over antibodies in easy synthesis, low cost, good chemical stability, and ease of labeling functional groups (Li et al., 2015; Song et al., 2008; Tombelli et al., 2005). Since the discovery of anti-BPA aptamers (Jo et al., 2011), some aptamer-based assays have been developed for the detection of BPA with different transduction methods, such as fluorescence (Yun et al., 2018), electrochemistry (Shi et al., 2018), surface-enhanced Raman (Chung et al., 2015), photoelectrochemistry (Wang et al., 2018), and etc (Ragavan et al., 2013). Among these methods, the fluorescence assays have strengths in high sensitivity and easy operation.

Fluorescence anisotropy (FA) assay is a reliable fluorescence method based on the change in the rotational rates of the labeling fluorophores in the binding events. FA assay is a ratiometric method, so it is insensitive to the fluorescence fluctuation and photo-bleaching, showing advantages in good reproducibility and simplicity. FA analysis is widely applied in many fields such as molecular interaction study, drug discovery, assays in clinical test and environmental analysis, and etc (Hall et al., 2016; Jameson and Ross, 2010). Taking advantages of the aptamers, aptamer-based FA methods have emerged for small molecules detection, attracting wide attentions (Liu et al., 2013; Ruta et al., 2009; Zhang et al., 2019; Zhao et al., 2014; Zhao et al., 2015; Zhao et al., 2019). Among the aptamer-based FA assays, direct FA assays using one fluorescently labeled aptamer probe are much simpler than those competitive FA methods using more than one reagent (Zhang et al., 2019; Zhao et al., 2019). The direct aptamer FA assay is based on the unique binding induced conformation change of aptamer and alteration of FA signals (Ruta et al., 2009; Zhao et al., 2014; Zhao et al., 2015). Our group previously developed aptamer-based direct FA assays for small molecules using the tetramethylrhodamine (TMR)-labeled aptamers. In these assays, the binding events induced the conformation change of aptamers and the alteration of the interaction between TMR and guanine (G) bases, which generated significant FA changes (Zhao et al., 2014; Zhao et al., 2015). Aptamer FA assays for BPA have not been reported yet.

In this work, taking advantages of aptamer and fluorescence anisotropy analysis, we describe a direct aptamer FA method for BPA detection by using a single TMR labeled 35-mer aptamer probe. We labeled single TMR on a specific site of the aptamer, including the thymine (T) bases and the terminals of the aptamer. We tested the FA responses of a series of TMR-labeled aptamer probes to BPA. To evaluate the effect of TMR labeling sites on the binding affinity of aptamer, we measured the dissociation constants (K_ds) of the TMR-labeled aptamer probes with microscale thermophoresis (MST), which is a powerful technique for affinity binding study (Entzian and Schubert, 2016). In our FA assay, the aptamer labeled TMR in the 22nd T base showed largest FA change induced by BPA-binding and maintained good binding affinity and generated signal-decreasing response. We further investigated the effects of experimental conditions including the metal cations in binding buffer solution. A simple and rapid aptamer-based FA assay for BPA detection was achieved under the optimized condition. BPA spiked in tap water sample can be detected, showing the FA assay has potential to be applied in water sample analysis.

Table 1 – List of aptamer probes with TMR labeling at different sites.

Name	Sequence
BP35-5′-TMR	5′-(TMR)-CCGCCGTTGGTGTGGTGGGCCTAGGGCCGGCGG-3′
BP35-7T-TMR	5′-CCGCCGT-(TMR)TGGTGTGGTGGGCCTAGGGCCGGCGG-3′
BP35-8T-TMR	5′-CCGCCGTT-(TMR)GGTGTGGTGGGCCTAGGGCCGGCGG-3′
BP35-11T-TMR	5′-CCGCCGTTGGT-(TMR)GTGGTGGGCCTAGGGCCGGCGG-3′
BP35-13T-TMR	5′-CCGCCGTTGGTGT-(TMR)GGTGGGCCTAGGGCCGGCGG-3′
BP35-16T-TMR	5′-CCGCCGTTGGTGTGGT-(TMR)GGGCCTAGGGCCGGCGG-3′
BP35-22T-TMR	5′-CCGCCGTTGGTGTGGTGGGCCT-(TMR)AGGGCCGGCGG-3′
BP35-3′-TMR	5′-CCGCCGTTGGTGTGGTGGGCCTAGGGCCGGCGG -3′-(TMR)

The labeling position is shown in underline format.

1. Materials and methods

1.1. Chemicals and materials

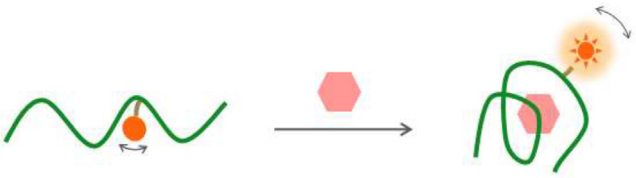
Bisphenol A (BPA), tyrosine (Tyr), arginine (Arg), ampicillin (Amp), and tetracycline (Tet) were purchased from Sigma. Bisphenol S (BPS) and 4,4′-dihydroxybiphenyl (BP) were ordered from J&K Chemical (Beijing, China). All DNA oligonucleotides were synthesized and purified by Sangon Biotech (Shanghai, China). All other reagents were of analytical grade. Ultrapure water used in this experiment for solution preparation was obtained from a Purelab Ultra Elga Labwater system. The 35-mer aptamers with TMR labeling at different labeling sites are listed in Table 1.

1.2. Fluorescence anisotropy measurement

For fluorescence anisotropy (FA) measurements, fluorescence spectrometer (JASCO FP-8300, Japan) equipped with a thermostat for temperature control was employed. The fluorophores were excited by polarized light, and emitted light was collected from channels that are parallel and perpendicular to the electric vector of the excitation light. FA value is defined as $FA = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I corresponds to the emission intensity, and the subscripts V and H mean the vertical and horizontal orientation of the polarizer, respectively. G factor, which is given by $G = I_{HV}/I_{HH}$, is used for instrumental correction between different instruments. FA analysis was performed with an excitation at 550 nm and an emission at 580 nm. Slits for the excitation and emission were both set at 5 nm. Without otherwise statement, the measurements of FA analyses were conducted at 25°C. For FA detection of BPA, 20 nmol/L TMR labeled aptamer probes were incubated with varying concentrations of BPA for 15 min in the binding buffer containing 25 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, and 10 mmol/L $MgCl_2$. Each sample was tested for five times and the average value was used for further data processing.

1.3. Microscale thermophoresis analysis

The MST experiments were performed on Monolith NT.115 instrument (Nanotemper, Germany) at room temperature. A green excitation light (510–550 nm) was chosen for TMR-labeled aptamer probes. MST power was set at medium level. LED power was set at auto mode. Sixteen samples with constant concentration of TMR-labeled aptamer (50 nmol/L) and varying concentrations of BPA ranging from 100 μmol/L to 3.1 nmol/L were prepared. The MST binding buffer contained 25 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, and 10 mmol/L $MgCl_2$. After incubation for 10 min, the samples were loaded into 16 capillaries (NanoTemper Technologies). Each capillary was successively scanned by the instrument.



Scheme 1 – Principle of fluorescence anisotropy assay for BPA with TMR-labeled aptamer. TMR is labeled on a specific position of the aptamer against BPA. The BPA binding induces the conformation change of aptamer and the alteration of the intramolecular interaction between TMR and ambient G bases, thus causing remarkable FA change.

Initial fluorescence was scanned for 5 sec to ensure homogeneity before switching the IR heating laser on. Then, the activation of the IR laser generated a microscopic temperature gradient of 2–6°C, causing the directional movement of aptamers and aptamers-BPA complex. During thermophoresis, unbound probe and aptamer-BPA complex showed different behaviors. After about 20 sec, IR laser was off. A series of MST traces were recorded for each sample. After three times measurements, the average value and the standard deviation were used. With the MO affinity analysis software, the recorded fluorescence signal was normalized to F_{norm} (F_{hot}/F_{cold}, F_{cold} is the fluorescence value measured before turning the IR laser on, F_{hot} is the fluorescence value measured in the MST-on time) and fraction bound (0 = unbound, 1 = bound), then dissociation constants (K_ds) of the aptamer probes to BPA were derived by using the law of mass action.

2. Results and discussion

2.1. *Screening FA responses of TMR-labeled aptamers to BPA*

Scheme 1 shows the principle of FA assay for BPA detection by using a single TMR-labeled aptamer. It is reported that the interaction between TMR and ambient G bases in aptamer causes the fluorescence quenching through photoinduced electron-transfer (PET) and affects the FA value by restricting the local rotation of TMR (Unruh et al., 2005; Zhang et al., 2012). When BPA binding to aptamer, an adaptive conformation of aptamer is induced, thus the intramolecular interaction between TMR and ambient G bases possibly alters, causing changes of FA signals. If the BPA-aptamer binding weakens the interaction, TMR shows more local rotation freedom, leading to lower FA value. While a higher FA value will be observed when the TMR-G interaction is strengthened. Therefore, BPA can be detected by measuring FA change of TMR. A proper site on the aptamer for TMR labeling is critical for the sensitive detection of BPA.

We conjugated single TMR on different sites of a 35-mer aptamer against BPA, which was truncated from a long sequence of aptamer (Jo et al., 2011; Marks et al., 2014). The screened labeling sites included 5′-terminal, 3′-terminal, and internal thymine (T) bases (7T, 8T, 11T, 13T, 16T, 22T) as shown in Table 1. We measured the FA values of TMR labeled aptamers before and after BPA addition. In the absence of BPA, most of TMR labeled 35-mer aptamer probes showed high FA values of higher than 0.210, while the FA value of BP35-13T-TMR was about 0.168 (Fig. 1a). In the presence of 50 μmol/L BPA, the FA-responses of TMR-labeled aptamers upon BPA were remarkably different (Fig. 1b). The BP35-7T-TMR, BP35-11T-TMR, BP35-13T-TMR showed negligible FA response upon BPA. The BP35-5′-TMR exhibited FA increase, which was different from the other aptamer probes. It suggests that the BPA binding tightens the intramolecular interaction between G bases and TMR labeled at 5′ end of anti-BPA aptamer (Zhao et al., 2015). The BP35-8T-TMR, BP35-16T-TMR, and BP35-3′-TMR showed slight FA reduction with the addition of 50 μmol/L BPA. BP35-22T-TMR is a more FA-responsive probe among these TMR-labeled aptamer, and the presence of 50 μmol/L BPA caused FA decrease about 0.024. When fluorescein (FAM) was labeled on the 22nd T in the sequence, the FAM labeled aptamer did not show FA changes upon addition of BPA, which shows TMR-G interaction is specific. Therefore, we chose BP35-22T-TMR as a fluorescent probe in the FA assay.

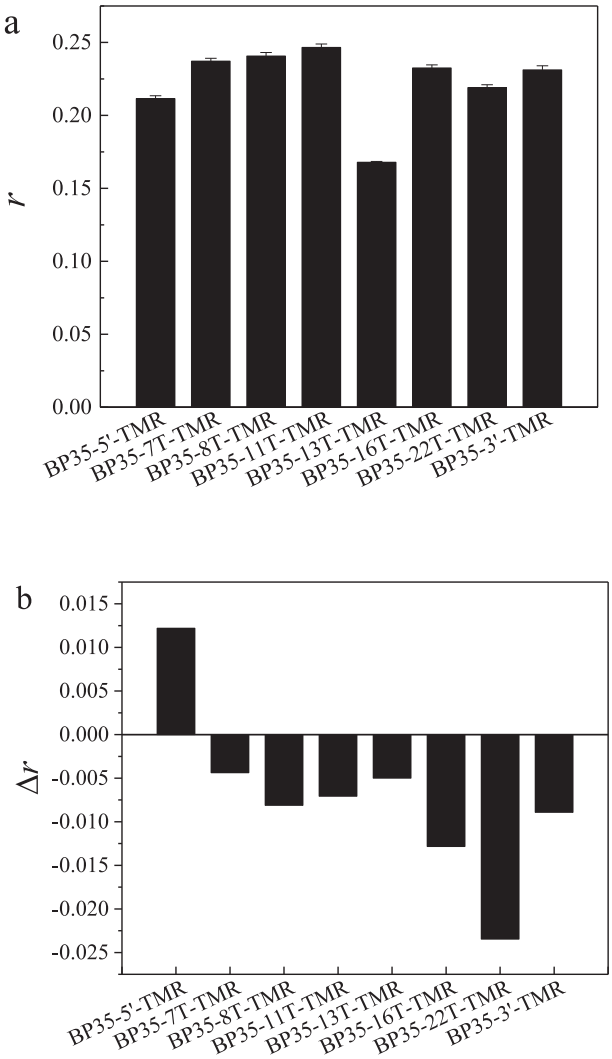


Fig. 1 – The FA (r) value of TMR labeled aptamers (a) and the FA change (Δr) in the presence of 50 μmol/L BPA (b). Binding buffer: 25 mmol/L Tris–HCl (pH=8.0), 150 mmol/L NaCl and 10 mmol/L $MgCl_2$. The concentration of TMR labeled aptamers was 20 nmol/L.

2.2. *Characterization of TMR labeled aptamers with microscale thermophoresis*

To test whether the insignificant FA change of some TMR-labeled aptamers upon binding to BPA are caused by the binding affinity decrease or the slight change of intramolecular

Table 2 – Binding affinity of TMR-labeled aptamers determined by MST analysis.

Name	K_d (μmol/L)
BP35-5′-TMR	8.0±2.3
BP35-7T-TMR	NB
BP35-8T-TMR	129±34
BP35-11T-TMR	NB
BP35-13T-TMR	NB
BP35-16T-TMR	91±28
BP35-22T-TMR	17±6.5
BP35-3′-TMR	10±4.9

NB means no binding affinity.

interaction between TMR and G bases, we further used microscale thermophoresis (MST) analysis to characterize the binding affinity of TMR labeled aptamer probes to BPA (Appendix A **Fig. S1**, Table 2).

MST is a powerful tool to study biomolecular interactions by monitoring the movement of fluorescently labeled molecules in temperature gradients (Entzian and Schubert, 2016; Jerabek-Willemsen et al., 2011). The interactions between some small molecules and aptamers have been successfully characterized by MST (Entzian and Schubert, 2016). In MST analysis, the binding of target to fluorescently labeled ligand causes changes in charge, size, or hydration shell and alteration of thermophoretic mobility of fluorescently labeled ligand through a microscopic temperature gradient, leading to the variance of fluorescence intensity of fluorescently labeled ligand in the testing zone (Entzian and Schubert 2016; Jerabek-Willemsen et al., 2011). The measurement of MST traces for a series of samples with fluorescently labeled ligand at constant concentration and varying concentrations of targets allows for the characterization of the binding affinity.

Appendix A **Fig. S1** shows the results of MST analysis of binding affinity of TMR-labeled aptamers. The normalized fluorescence (F_{norm}) of MST traces was plotted against the concentrations of BPA. The curves of TMR-labeled aptamers start from different F_{norm} and show different amplitudes (Appendix A **Fig. S1a-h**). Fraction bound was plotted against the concentrations of BPA to compare the binding affinity of TMR-labeled aptamers which have MST response to BPA (Appendix A **Fig. S2**).

Table 2 summarizes the dissociation constants (K_ds) of TMR-labeled aptamers determined by MST. MST analysis did not get binding affinity of BP35-7T-TMR, BP35-11T-TMR and BP35-13T-TMR to BPA, so the negligible FA responses of these three probes to BPA were possibly due to the affinity loss. BP35-8T-TMR and BP35-16T-TMR exhibited greatly reduced binding affinity, and their FA changes to BPA were not large. BP35-5′-TMR, BP35-3′-TMR and BP35-22T-TMR maintain good binding affinity to BPA, with the dissociation constant at 8.0±2.3 μmol/L, 10±4.9 μmol/L and 17±6.5 μmol/L, respectively. However, BP35-3′-TMR did not show remarkable FA response to BPA, meaning it lacked large alteration of the local rotation of TMR and the TMR-involved intramolecular interaction upon BPA binding.

2.3. *Optimization of FA assay for BPA*

To figure out the optimum conditions for FA assay, we investigated the effects of experimental conditions on FA responses of BP35-22T-TMR to BPA, including the concentrations of $MgCl_2$, NaCl, and KCl in binding buffer solution. Under the different conditions, we monitored the FA value of BP35-22T-TMR (20 nmol/L) in the absence of BPA and the FA change upon 100 μmol/L BPA.

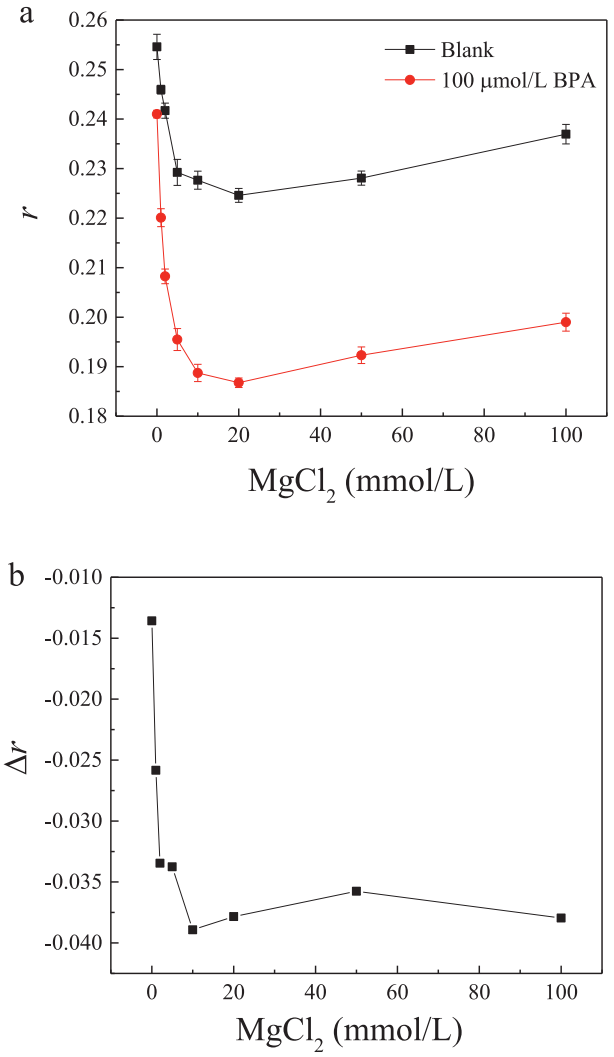


Fig. 2 – The effects of $MgCl_2$ on FA responses of BP35-22T-TMR. (a) FA value of 20 nmol/L BP35-22T-TMR in the absence or the presence of 100 μmol/L BPA, (b) FA change (Δr) caused by 100 μmol/L BPA. The binding buffer contained 25 mmol/L Tris–HCl (pH = 8.0), 100 mmol/L NaCl, and varying concentrations of $MgCl_2$.

We first tested the effect of $MgCl_2$ in binding buffer. As shown in Fig. 2**a**, in the absence of BPA, the FA value of BP35-22T-TMR sharply decreased from 0.255 to 0.225 and then slowly increased with addition of $MgCl_2$. It indicates that the addition of $MgCl_2$ may affect the conformation of aptamer and weaken the intramolecular interaction between TMR and ambient G bases. The absolute FA change (Fig. 2**b**) of BP35-22T-TMR upon BPA binding was negligible when the binding buffer did not contain $MgCl_2$. With the addition of $MgCl_2$, the absolute FA change remarkably increased. The result suggests $MgCl_2$ is important for FA sensing BPA with BP35-22T-TMR, which may help the aptamer to maintain suitable structure for affinity binding. When the concentration of $MgCl_2$ was higher than 10 mmol/L, the absolute FA change became smaller. Thus the addition of 10 mmol/L $MgCl_2$ in the binding buffer was applied in subsequent experiments.

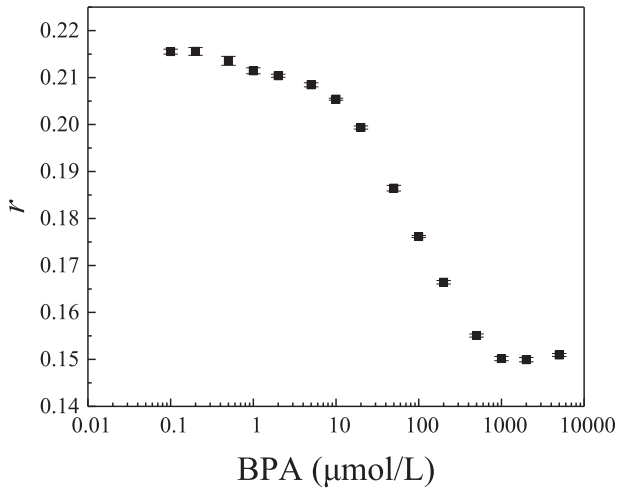


Fig. 3 – Direct FA assay of BPA using aptamer probe BP35-22T-TMR.

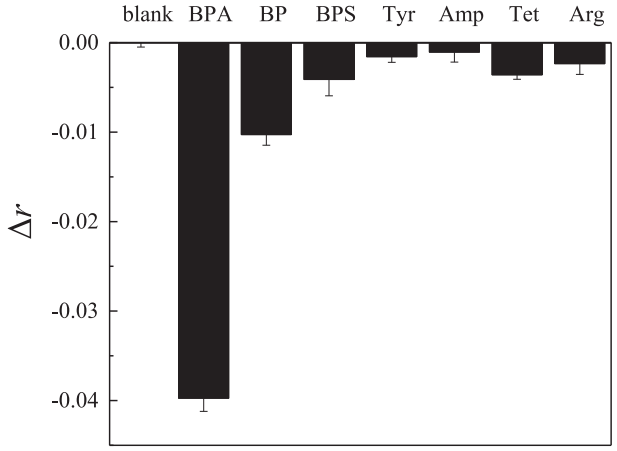


Fig. 4 – Selectivity of FA assay using BP35-22T-TMR for BPA detection. FA change (Δ*r*) of BP35-22T-TMR were tested in the absence of BPA (blank) and in the presence of 100 μmol/L BPA, 100 μmol/L 4,4′-Dihydroxybiphenyl (BP), 100 μmol/L bisphenol S (BPS), 100 μmol/L tyrosine (Tyr), 100 μmol/L ampicillin (Amp), 100 μmol/L tetracycline (Tet), or 100 μmol/L arginine (Arg).

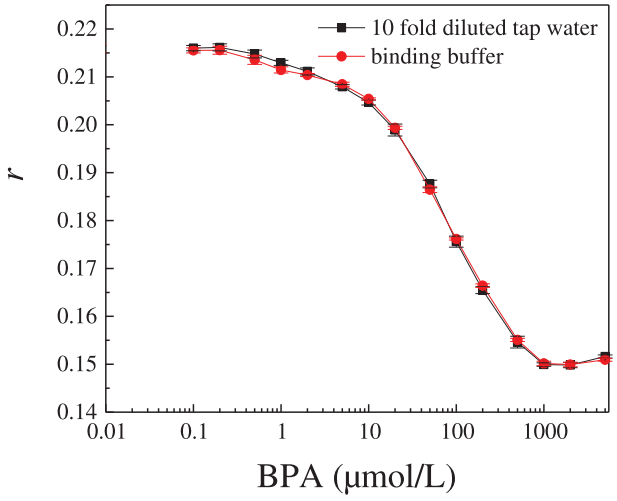


Fig. 5 – FA assay of BPA spiked in 10-fold diluted tap water.

The concentration of NaCl affected the FA value of BP35-22T-TMR and the FA change to BPA (Appendix A **Fig. S3**). The FA value of BP35-22T-TMR decreased with the increase of NaCl when BPA was absent. When BPA was present, 150 mmol/L NaCl in binding buffer was favorable for large FA response. We further tested the influence of KCl in the solution containing 10 mmol/L $MgCl_2$ and 150 mmol/L NaCl (Appendix A **Fig. S4**). The absolute FA change caused by BPA gradually reduced with the addition of KCl. It shows that KCl is not preferred for large FA changes caused by BPA. Thus, we did not add KCl in the binding buffer in the following experiments.

2.4. *Analytical performance of the method*

Under the optimal conditions, the FA assay of BPA using BP35-22T-TMR was achieved (Fig. 3). With the addition of BPA, the FA value gradually reduced. The detection limit of BPA was 0.5 μmol/L which was determined by the signal to noise ratio > 3. In the concentration range from 5 μmol/L to 1000 μmol/L, the FA values showed a linear relationship with the logarithm of concentration of BPA (y = -0.0276lg$x$ ++0.2316, R^2 = 0.9882). The dynamic detection range was from 0.5 to 1000 μmol/L, covering about three orders of magnitude. The relative standard deviations for five repeated analyses were less than 3%.

Compared with some reported aptamer-based detection methods for BPA, the sensitivity of our assay is lower (Guo et al., 2018; Marks et al., 2014; Ragavan et al., 2013; Shi et al., 2018; Su et al., 2018; Wang et al., 2018; Zhu et al., 2015). The comparison with other reported aptamer based assays for BPA was summarized in Appendix A **Table S1**. However, our FA assay only uses a TMR-labeled aptamer, showing the merits of simplicity, rapidity, good reproducibility and the potential for high throughput analysis. Combining sample preconcentration, lower concentrations of BPA can be detected by using this FA assay. In addition, signal amplification strategy may help to improve the sensitivity of the FA assay. The use of anti-aptamer with higher binding affinity will also improve the sensitivity of this aptamer FA assay.

2.5. *Assay selectivity and performance in complex sample matrix*

The selectivity of the FA assay with BP35-22T-TMR for BPA detection was examined by using the analogues of BPA including bisphenol S (BPS), 4,4′-dihydroxybiphenyl (BP), a few amino acids and antibiotics including tyrosine (Tyr), arginine (Arg), ampicillin (Amp), and tetracycline (Tet). Among the tested compounds in a high concentration of 100 μmol/L, insignificant FA change of BP35-22T-TMR were observed, while the presence of BPA caused a large FA decrease (Fig. 4). The result shows the FA assay is selective for BPA detection.

To evaluate the feasibility of FA analysis of BPA detection in actual water samples, we used tap water sample as an example. BPA spiked in 10-fold diluted tap water sample was tested by using BP35-22T-TMR. The quantification curves of BPA detection in the binding buffer and tap water were similar (Fig. 5). The recovery rates of 5 μmol/L, 50 μmol/L and 500 μmol/L spiked BPA in the diluted tap water sample were 104%, 95%, and 100%, respectively. Because metal ions (e.g., Mg^{2+}, Na^{+}, K^{+}) can have effect on the FA signal in the assay (Fig. 2, Appendix A **Fig. S3** and **Fig. S4**), the water sample should be diluted with the optimized binding buffer to reduce possible effects from sample matrix prior to analysis. The result indicates that the FA assay using BP35-22T-TMR has potential to detect BPA in actual water samples.

3. Conclusions

In summary, we developed a direct aptamer-based FA assay for BPA detection using a single TMR labeled 35-mer aptamer against BPA. After screening different labeling sites on aptamer, we identified that when TMR labeled on 22nd T base, the TMR-labeled aptamer showed remarkable FA change in the presence of BPA. Under the optimized condition, BP35-22T-TMR probe allowed to determine BPA with a detection limit of 0.5 μmol/L and good selectivity. This method has the advantages of simplicity and rapidity, and shows the potential application for water sample analysis.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.04.016.

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