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A novel pyrene-switching aptasensor for the detection of bisphenol A

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BisphenolA (BPA) is an environmental endocrine disrupter which is highly harmful to human health. Although a number of traditional analysis methods, such as high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), enzyme linked immunosorbent assay (ELISA) and other biochemical tests, have been used for the measurement of BPA, these analyses are limited by the availability of commercial antibodies, need for sophisticated equipment and tedious prior sample treatment. To address these limitations, we report herein the design, synthesis and application of a competition-mediated pyrene-switching aptasensor for the selective detection of BPA in buffer and actual water samples. The detection principle is based on the attachment of pyrene molecules to both ends of a hairpin DNA strand, which becomes the partially complementary competitor to an anti-BPA aptamer. The fact that two pyrenes in close proximity to each other can form an excimer plays an important role in this method. Triggered by BPA, the molecular probe can form a hairpin structure, resulting in a fluorescence emission shift from 400 nm (pyrene monomer) to 488 nm (pyrene excimer). This method exhibits a linear detection range from 0 to 2.00 ng mL^{-1} for BPA with a detection limit of 0.094 ng mL^{-1} (3σ). In addition, the method has high selectivity for BPA and can be effectively applied to actual environmental water samples.

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1. Introduction

Bisphenol A (BPA) is a chemical known as an environmental endocrine disruptor. It is an important industrial chemical in the production of polycarbonate plastic, epoxy and many plastic consumer products, including toys, water pipes, drinking water containers, spectacle lenses, sports safety equipment, dental monomers, medical equipment, pipelines and consumer electronic products.[1–4] Human and animal exposure to numerous sources of BPA can pose a serious threat to human health. Trace amounts of BPA can lead to dizziness and behavioural change. In addition, animal studies have shown that BPA also affects the prostate function.[5] Moreover, a direct mitogenic effect of BPA has been demonstrated in explant studies.[6] Accordingly, the detection of BPA in the environment is particularly important. Traditionally, high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), enzyme linked immunosorbent assay (ELISA) and other biochemical analysis techniques are among the most used methods for BPA analysis.[7–10] However, these assays are limited by the availability of commercial antibodies, need for sophisticated equipment and tedious experimental procedures. Therefore, the establishment of a method that involves a simple and mild reaction, and with high sensitivity and good selectivity for BPA analysis is still necessary and valuable.

An aptamer is an oligonucleotide fragment that can be folded into a unique three-dimensional conformation and which can be obtained from nucleicacid molecular libraries using *in vitro* screening techniques, such as SELEX (systemic evolution of ligands by exponential enrichment).[11,12] Aptamers have the advantages of small molecular weight, high stability, easy modification, easy synthesis, easy fixation, reusability and long-term preservation.[13–20] To date, they have also been widely used in therapeutics, diagnostics, research and biosensors.[21–24] As reported in the literature, aptamer-based strategies can efficiently provide simplicity and speed combined with satisfactory selectivity. Recently, an aptamer sequence of BPA was obtained and successfully used in the electrochemical detection of BPA in milk products and water samples.[25,26] This is very important for the construction of novel aptasensors for the detection of BPA.

The pyrene molecule is one of the most interesting organic dyes, which has the advantage of versatility for various chemical modifications.[27–29] Pyrene-based fluorophores have large extinction coefficients, excellent quantum yields, ~60 to 100 ns lifetimes, and good stability in aqueous solution. Pyrene also forms an excited state dimer, termed an excimer, with readily detectable emission that is red shifted by approximately 100 nm relative to the monomer. This emission wavelength switching

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can solve the probe background signal problem that occurs with FRET molecular probes. Bis-pyrene-labeled oligonucleotides have been widely used to probe DNA duplex formation and RNA folding by monitoring the monomer and excimer emission fluctuations.[30–32]

Motivated by these advantages of aptamers and the pyrene fluorophore, we report herein a novel fluorescence aptasensor for BPA detection by combining pyrene fluorophores and the aptamer of BPA. First, the bis-pyrene-labeled hairpin-structured DNA probe, used as the competitor, was partially hybridized with the aptamer of BPA to form the aptasensor of BPA in the "off" state. After triggering by BPA, a pyrene-associated excimer can be formed accompanied by the formation of a hairpin structure, thereby emitting excimer complex fluorescence and letting the pyrene fluorescence signal change to 488 nm (pyrene excimer). The proposed method has high sensitivity and selectivity, and was used to detect low concentrations of BPA and effectively applied to measure BPA concentration in actual environmental water samples.

2. Experimental section

2.1 Materials and reagents

The oligonucleotide sequences used in the experiments are as follows: the competitor: 5′-(pyrene) CCGGTGGGCCTGACCACCC ACCGG (pyrene)-3′; aptamer: 5′-CCGGTGGGTGGTCAGGTGGGA TAG CGTTCCGCG TAT GGCCCAGCG CAT CACGGGTTCG-CACCA-3′. The competitor was purchased from Takara Biotechnology (Dalian, China); the aptamer of BPA was purchased from Sangon Biotech (Shanghai, China). The purchased oligonucleotide strand was dissolved in ultrapure water and stored at −20 °C. BPA was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Bisphenol B (BPB), benzophenone, benzidine, dibenzoylhydrazide, catechol and resorcinol were purchased from Sinopharm Chemical Reagent Co., Ltd (Guangdong, China). BPA and all other reagents were of analytical grade and used without further purification. The actual water samples used in the application experiments were obtained from Peach Lake, Xiangjiang River (near the Hunan University) and tap water from Changsha city. The water used in all experiments was ultrapure water with a resistivity of 18.3 MΩ cm. Phosphate buffer (PBS) of different pH was prepared in advance by mixing different quantities of $Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4.

2.2 Instrumentation

All of the fluorescence measurements were performed on a fluorometer model FluoroMax-4 Spectrofluorometer (HORIBA Jobin Yvon Co., SAS, Longjumeau, France) and controlled by a personal computer. A quartz cuvette was the sample carrier used in the experiments. The pH meter used to detect the pH was a pHS-3B meter (Analytical Instruments, Shanghai, China). To prevent contamination of viruses or other organisms, the centrifuge tubes, buffer solution, pipette tips, and beakers were all sterilized by high pressure steam in a LDZX-30FBS vertical heating pressure steam sterilizer (ShenA Medical Instrument Factory, Shanghai, China). A model TE124s electronic balance (Sartorius Scientific Instrument Co., Ltd, Beijing, China) and a model TGL-16 centrifuge (Anting Scientific Instrument Factory, Shanghai, China) were also used to carry out the experiments.

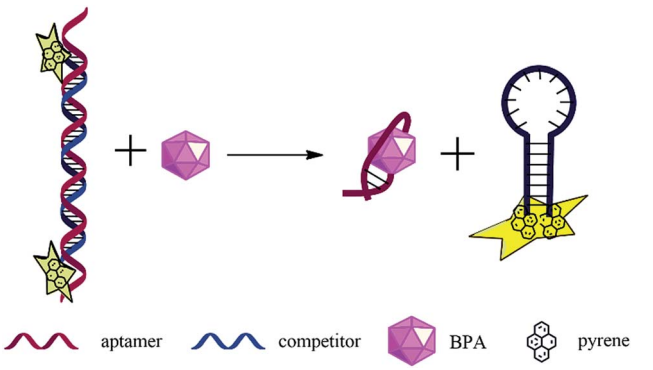
2.3 Preparation of the sensing system and fluorescence measurements

The mixed solution of competitor/aptamer, competitor and aptamer were activated at 65 °C for 5 minutes, and then diluted to 100 nM and stored at −20 °C for future use as a stock solution. After the preparation was completed, the competitor (2.5 μM, 15 μL) and aptamer (2.5 μM, 20μL) were added into a centrifuge tube containing 280 μL of PBS (pH 7.0), mixed at room temperature and allowed to hybridize for a few minutes, followed by the addition of the targets (100 ng mL^{-1}, 35 μL). Then, the mixtures were incubated for 15 minutes. Eventually, the fluorescence intensity was measured on the fluorometer mentioned above. The fluorescence intensity data reported in the figures of this article were all recorded at 488 nm.

3. Results and discussion

3.1 Detection principle of the pyrene-switching aptasensor

The schematic of this method is shown in Scheme 1. Two pyrene molecules were labeled at both the 3′ and 5′ends of the competitor, this competitor can be partially complementary to the aptamer,[33] and the standard equilibrium constant (K_m) value for the aptamer and competitor complex is *ca.* 6.6×10^{25} calculated from their Gibbs free energy, indicating the high stability of the hybridization complexes. So, in the absence of BPA, the competitor was conjugated to the BPA aptamer and the two pyrene molecules were far away from each other, while in the presence of BPA the aptamer detached from the competitor and preferred to associate with BPA. The released competitor re-adopts its hairpin structure (the standard equilibrium constant of the hairpin is *ca.* 6.3×10^6), so that the two pyrene molecules are close to each other to form an excimer that emits fluorescence.



Scheme 1 Working principle of the competitor/aptamer to construct an aptasensor to test BPA.

3.2 Optimization of the experimental conditions

3.2.1 Optimization of pH. Usually, the pH has a strong influence on aptamer and nucleic acid base pairing,[34] and the fact that pH can affect the fluorescence intensity of a fluorescent dye also cannot be ignored.[35] Therefore, it is necessary to determine the optimum pH for the experimental reaction to produce the maximum fluorescence intensity. Accordingly, the pH values of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 were selected for this experiment. The results of this experiment are shown in Fig. 1(A). The figure reveals that the fluorescence signal increased in the pH range of 6.0 to 8.0 and decreased in the pH range of 8.0 to 9.0. Since we detected the strongest fluorescence signal intensity at pH 8.0, the pH value of 8.0 was chosen as the optimized pH for this experiment.

3.2.2 Optimization of incubation time. In general, the longer the incubation time, the more stable the double helix structure can be. However, when the hybridization time is too long, it would consume time and non-specific combination might occur.[36,37] The effects of hybridization time on the efficacy of the method were investigated for two processes. The fluorescence intensity of the first process was detected after the aptamer and competitor were hybridized. Meanwhile, the second process was carried out under the conditions that the optimum pH and optimum hybridization time of the aptamer with the competitor were achieved, then a new optimal experiment was conducted to detect the optimum wrapping time. The results are shown in Fig. 1B and C. The response time of the aptamer to the competitor was very fast (3 minutes), and the hybridization time of the aptamer with the competitor had little impact on the experimental results after 3 minutes (Fig. 1B). After adding BPA, the fluorescence intensity sharply increased as the time increased from 2 to 20 minutes, and then reached a plateau (Fig. 1C). Accordingly, herein 3 minutes for the hybridization of the competitor with the aptamer and 25 minutes for associating with BPA were chosen as the suitable times for the optimized experimental conditions.

3.2.3 Optimization of incubation temperature. The reaction temperature is also a factor that should be considered. The temperature has an effect on the activity of DNA, thus it is important to study its effect on the experimental results. As shown in Fig. 1D, the temperature has little influence on the reaction in the range from 10 °C to 40 °C, while between 40 °C and 65 °C the signal gradually increased, but once the temperature exceeded 65 °C the signal decreased as time increased. This phenomenon may be explained by the release of the double helix structure when the temperature exceeds 40 °C, which allows the competitor to re-adopt its original hairpin structure, and the pyrene at both ends of the competitor then emits the excimer fluorescence, but, when the temperature exceeds 65 °C, the stem loop structure melts into an extended random coil.[38,39] So, 65 °C was chosen as the reaction temperature in the subsequent experiments.

3.3 Quantitative detection of BPA in pure buffer

The sensitivity of the method was evaluated after optimizing all possible parameters. The results are shown in Fig. 2. The concentration of BPA was controlled between 0 and 80.0 ng

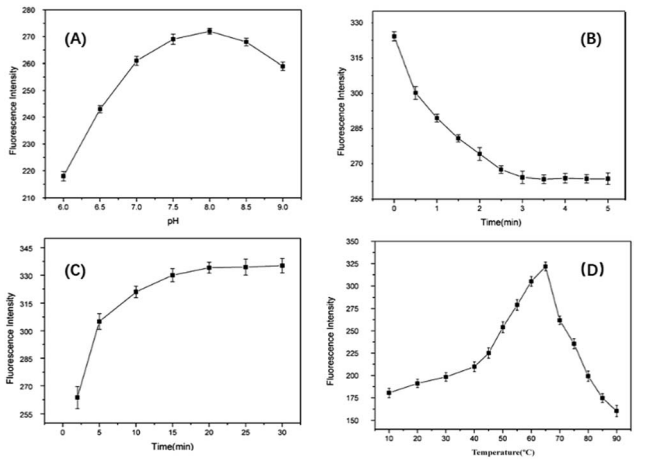


Fig. 1 (A) Effects of the pH value. The concentration of BPA was 8.00 ng mL^{-1}, the hybridization time was 2 minutes and wrapping time was 20 minutes. (B) Effects of the competitor and aptamer hybridization time. The concentration of BPA was 8.00 ng mL^{-1} and the pH was 8.0. (C) Effects of the incubation time. The concentration of BPA was 8.00 ng mL^{-1}, the pH was 8.0 and the competitor and aptamer hybridization time was 3 minutes. (D) Effects of the temperature. The concentration of BPA was 8.00 ng mL^{-1}, the pH was 8.0 and the hybridization time was 3 minutes and 20 minutes.

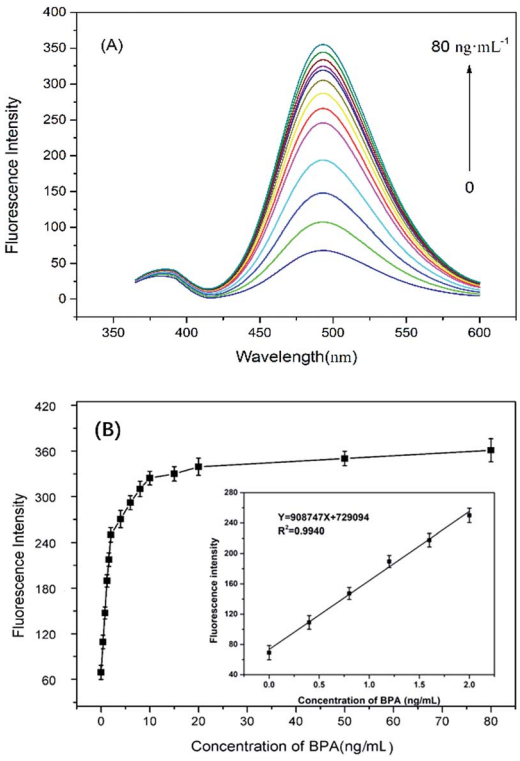


Fig. 2 Sensitivity of the proposed method: (A) fluorescence emission spectrum of the sensing system with different concentrations of BPA. (B) Plot of the fluorescence intensity which is dependent on the BPA concentration. The inset figure is the fluorescence intensity linear curve and linear regression equation for BPA. The data were recorded at the emission wavelength of 488 nm.

mL^{-1}, and the concentrations of 0, 0.40, 0.80, 1.20, 1.60, 2.00, 4.00, 6.00, 8.00, 10.0, 15.0, 20.0, 50.0 and 80.0 ng mL^{-1} were chosen for evaluation. Clearly, the fluorescence intensity at 488 nm increased with the increase of the BPA concentration (Fig. 2). The dependence of the fluorescence intensity on BPA concentration is plotted in Fig. 2B, the data reveal that high concentrations of BPA result in strong fluorescence intensity. In addition, a good linear relationship between the BPA concentration and fluorescence intensity was found in the range from 0 to 2.00 ng mL^{-1} (Fig. 2B). The detection limit was estimated to be 0.094 ng mL^{-1} according to the following eqn (1),

$$(3S + \bar{F} - b)/K \quad (1)$$

where S is the standard deviation of the blank control, K is the slope of the regression equation, $\bar{F}$ denotes the average background fluorescence intensity, and b is the intercept in the linear regression equation. This is a remarkably low detection limit for BPA detection using a fluorescence method which is much lower than the drinking water health standard level (GB5749-2006) – whose maximum level of BPA is 10 ng mL^{-1}. According to the data presented in Table 1, this method has a strong sensitivity compared with some other reported experimental data. Thus, this method can be used to detect BPA in the actual environmental water samples.

3.4 Selectivity of the aptasensor system

To evaluate the selectivity of this method, two control experiments were conducted. First, BPA and other chemicals including BPB, benzidine, dibenzoyl, hydroquinone and resorcinol (these chemicals are very similar in structure to BPA, their structures are shown in Fig. 3) were added separately to the sample solutions, and then the fluorescence intensity was recorded. As shown in Fig. 4, only the addition of BPA induced a strong fluorescence signal, whereas the addition of the other chemicals did not generate a significant increase in the fluorescence signal. These results demonstrate that the excellent selectivity for BPA is far higher than that for other chemicals. Second, another anti-interference experiment was conducted, by adding BPA and the other chemicals in a mixture sample solution for fluorescence testing, the results showed that no significant fluorescence signal is detected in the absence of BPA. Aptamers can form a specific secondary structure or tertiary structure and closely bind to the target, they can form

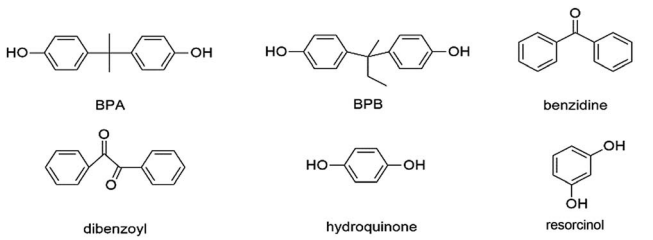


Fig. 3 Structures of the six chemicals used in the study to evaluate the selectivity for BPA.

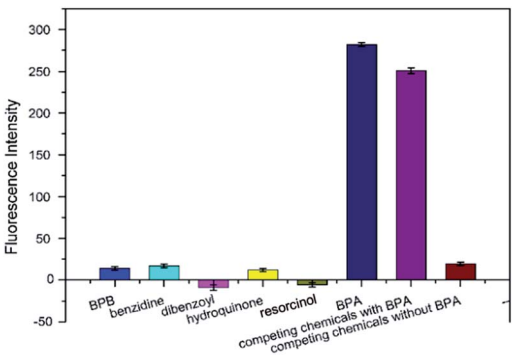


Fig. 4 Selectivity of this method. The concentration of BPA was 10 ng mL^{-1} and the concentrations of the five interfering chemicals (BPB, benzidine, dibenzoyl, hydroquinone and resorcinol) were 100 ng mL^{-1}.

some stable three-level spatial structures (convex, G-tetrad, hairpin, pseudoknot, *etc.*) through pairing, hydrogen bonding, van der Waals force, *etc.*, in some complementary bases in the chain, and they can also specifically bind to the target through these structures to form a stable complex; when the target is a small molecule, the aptamer folds itself to form a binding pocket, and wraps the target molecule inside.[40,41] These studies may explain why only BPA causes significant changes in the fluorescence signal. The high selectivity and anti-interference of the proposed method can help avoid false positive results in the testing of environmental water samples.

3.5 Measurement of BPA in a real water sample with the proposed aptasensor

Considering the high sensitivity and selectivity of this method, a standard addition method was used to establish whether the

Table 1 Comparison of the proposed method for BPA detection with other methods

Methods	Linear range (ng mL^{-1})	Detection limit (ng mL^{-1})	References
Immunoaffinity/HPLC/fluorescence	0.16–39.90	0.16	42
ELISA	1.62–820	2.28	43
Immunochromatographic assays	0–2.28	0.21	44
Immunoassay	20.8–330.3	13.1	45
Fluorescence (gold nanoparticles)	1.14–2280	0.456	46
Isotope dilution mass spectrometry	No	0.027	47
Fluorescence (silver nanocluster)	2×10^2 to 2×10^6	20	48
Fluorescence	0–2.00	0.094	This work

Table 2 BPA concentrations in environmental samples detected by the proposed method and HPLC analysis

Sample	Added BPA (ng mL^{-1})	The proposed method mean^a ± SD^b (ng mL^{-1})	Recovery	HPLC mean^a ± SD^b (ng mL^{-1})	Recovery
Tap water	0.00	0.120 ± 0.112		0.111 ± 0.021	
	0.40	0.414 ± 0.011	73.5 ± 25.3%	0.397 ± 0.134	71.5 ± 28.3%
	0.80	0.781 ± 0.134	82.6 ± 2.75%	0.804 ± 0.051	86.6 ± 3.75%
	1.20	1.212 ± 0.121	91 ± 0.75%	1.230 ± 0.010	93.3 ± 0.92%
Lake water	0.00	0.110 ± 0.056		0.145 ± 0.116	
	0.40	0.382 ± 0.101	68 ± 11.3%	0.357 ± 0.136	53 ± 5%
	0.80	0.810 ± 0.126	87.5 ± 8.75%	0.771 ± 0.074	78.3 ± 5.25%
	1.20	1.194 ± 0.052	90.3 ± 0.33%	1.198 ± 0.187	87.8 ± 5.92%
River water	0.00	0.160 ± 0.071		0.136 ± 0.014	
	0.40	0.413 ± 0.024	63.3 ± 11.8%	0.412 ± 0.128	69 ± 28.5%
	0.80	0.792 ± 0.115	79 ± 5.5%	0.821 ± 0.115	85.6 ± 12.6%
	1.20	1.240 ± 0.018	90 ± 4.42%	1.214 ± 0.071	89.8 ± 4.75%

^{a} Mean of three determinations. ^{b} SD, standard deviation.

proposed method was applicable to actual environmental water samples (tap water, river water and lake water). The river and lake water samples mentioned above were filtered using a needle-type filter to remove the particulates before testing. This was followed by addition of 0.40, 0.80 and 1.20 ng mL^{-1} BPA to the samples, three separate parallel samples were set up for different types of water of different concentrations; the linear equation was used to find the corresponding concentration value. The same spiking experiments were performed using HPLC (a traditional detection method for BPA). The preparation of the sample used for the proposed method was carried out following the steps described in Section 2.3 above. For the preparation of the samples used for HPLC, BPA was directly added at different concentrations from those of the samples mentioned above, other conditions corresponded to natural conditions without artificial control. The results were compared with those obtained by the proposed method. The comparison is shown in Table 2, which reveals that the sample with 0 BPA added shows values higher than 0 by both the fluorescence and HPLC methods in all three water specimens, this may be because 0 is not within the detection limit of this method and there may have been an effect on the results when there are other BPA analogs in the water samples. Nevertheless, the relatively satisfactory results shown in the table indicated that the method can be applied to the analysis of BPA in environmental water samples.

4. Conclusions

In summary, a simple method for BPA detection in water has been successfully developed by changing the distance between the two pyrenes labeled in the competitor. After triggering by BPA, the competitor detaches from the aptamer and forms a hairpin structure that induces an excimer fluorescence signal. This method can achieve a detection limit of 0.094 ng mL^{-1}, which is much lower than the limit of BPA in drinking water health standard. The proposed method has a high selectivity and anti-interference for other chemicals whose structures are similar to that of BPA. In addition, the developed method was also successfully applied for the detection of BPA in environmental water samples with satisfactory results.

Conflicts of interest

There are no conflicts to declare.

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