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An amplifying DNA circuit coupled with Mg^{2+}-dependent DNAzyme for bisphenol A detection in milk samples

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ABSTRACT

As bisphenol A (BPA) is harmful to human health, it is of great significance to develop a new method for BPA detection. Herein, we designed a BPA biosensor by integrating an amplifying DNA circuit with Mg^{2+}-dependent DNAzyme into the sensing system. The BPA-aptamer binding activated a DNA circuit for signal amplification based on toehold-mediated strand displacement. A catalytic Mg^{2+}-dependent DNAzyme was formed through synergistically DNA hybridization, which can cleave the dual-labeled substrate DNA into two segments. The separation of the fluorophore and quencher produces a high fluorescence response for BPA detection. This biosensor exhibited a superior sensitivity with a detection limit of 50 fM. The method is selective and robust, which can work even in milk samples with satisfactory accuracy. The biosensor analytical results were also verified by liquid chromatography coupled with mass spectrometry (LC–MS) and no obvious difference existed between the two methods.

1. Introduction

As an important component of epoxy resins and polycarbonate, bisphenol A (BPA) is widely used in food packaging and water containers (Rajabnejad et al., 2020). BPA is an endocrine disruptive compound (EDC) that can produce adverse effects on human health even at very low concentrations (Abnous et al., 2018; Yu et al., 2019). The tolerable daily intake (TDI) level of BPA set by the European Food Safety Authority is 4 μg/kg body weight per day (Zehani et al., 2015). In addition, many diseases are related to BPA intake, such as reproductive and immune disorders, tumor progression, and various types of cancers (Allsop et al., 2019). Therefore, design of reliable and sensitive methods for BPA detection is important for protecting food safety and human health.

Traditional methods for BPA detection include high-performance liquid chromatography (HPLC), gas chromatography coupled with mass spectrometry (GC–MS), and liquid chromatography coupled with mass spectrometry (LC–MS) (Dreolin et al., 2019; Xiao et al., 2020). The challenge of those accurate methods is that they require expensive instruments, complicated pretreatment procedures, and highly skilled operators. As alternatives to traditional methods, some groups have developed ingenious biosensors for BPA detection using aptamer as the recognition sequence, such as electrochemical (Li et al., 2019; Ma et al., 2017; Shi et al., 2018), fluorescent (Chen & Zhou, 2016; Li et al., 2016; Zhu et al., 2018), and colorimetric sensing system (Jia et al., 2020; Lee et al., 2019). To obtain a high sensitivity, protein enzymes are often utilized in those sensors to catalyze the signal amplification process (Chen & Zhou, 2016; Li et al., 2016; Shi et al., 2018; Zhu et al., 2018). The poor stability of protein enzymes may affect their catalytic activities in different reaction temperatures. Thus, it is urgent to develop an enzyme-free detection method for BPA analysis with improved sensitivity.

Toehold-mediated DNA strand displacement reaction is proved to be a powerful tool for the construction of enzyme-free signal amplification format in sensor design (Simmel et al., 2019; Xiao et al., 2019; Zhang & Seelig, 2011). The strand displacement can be started at a short overhanging single-stranded fragment (referred to as toehold) and progresses through toehold binding, branch migration, and strand separation (Irmisch et al., 2020; Wu et al., 2020). The toehold DNA displacement exhibits some advantages, such as enzyme-free reaction, environmental robustness, efficient signal amplification capability, and easy scalability (Guo et al., 2020; Li et al., 2020; Ma et al., 2019). The flexible use of this simple and robust mechanism makes it possible to fabricate logic gates (Chen et al., 2015, 2018; Tian et al., 2020), catalytic

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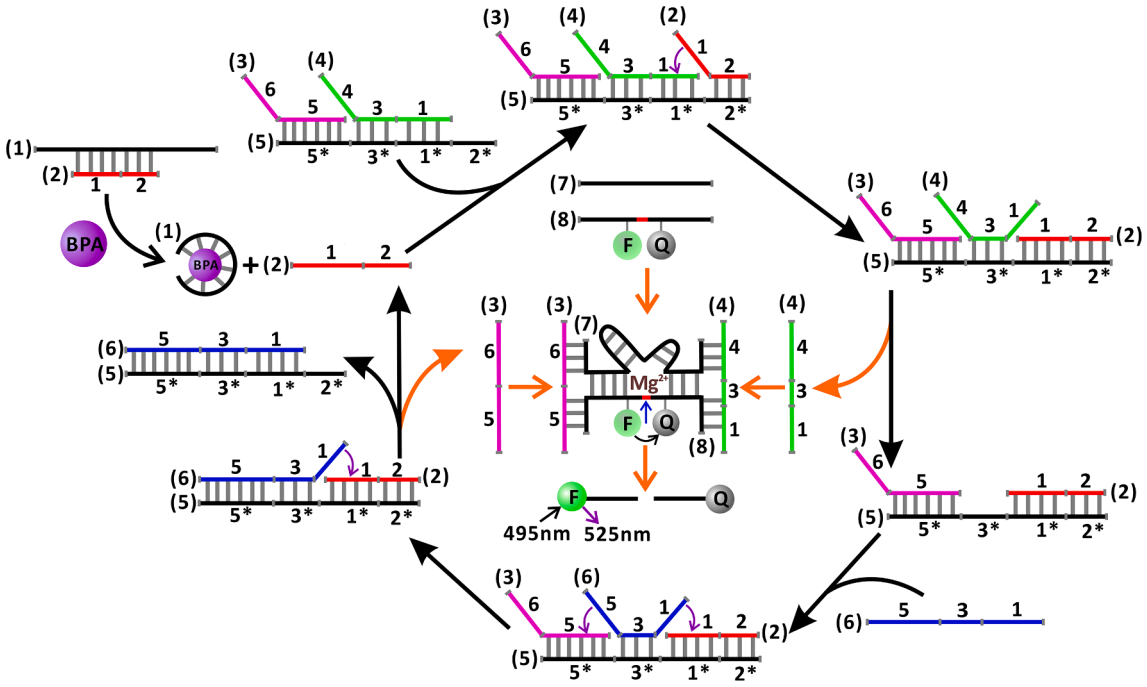

Fig. 1. Schematic diagram of an amplifying DNA circuit biosensor for bisphenol A detection. Number labels denote functional domains in the DNA probes. Domain x is complementary to domain x*.

amplifiers (Wang et al., 2018; Zhang et al., 2020a, 2020b; Zhu et al., 2020), molecular walkers (Oishi & Saito, 2020; Yao et al., 2020), and even neural networks (Cherry & Qian, 2018). To extend new applications of the toehold-mediated strand displacement, we constructed a fluorescent biosensor for BPA detection by integrating an amplifying DNA circuit with the Mg^{2+}-dependent DNAzyme (Chen et al., 2020; Yang et al., 2019) into the sensing system. The anti-BPA aptamer was used as the molecular recognition probe (Abnous et al., 2018; Yu et al., 2019). Through a series of displacement reactions, we have achieved a highly sensitive sensing system for nonenzymatic detection of BPA. The detection limit of our constructed biosensor is 50 fM and the detection linear range is from 100 fM to 1 μM. The assay is selective and robust, which can work even in milk samples with reliable results compared with LC–MS method.

2. Experimental section

2.1. Materials and reagents

All reagents were of analytical grade and obtained from Sigma-Aldrich (St. Louis, Mo, USA). All HPLC-purified sequences were purchased from Sangon Biotech Co., Ltd (Shanghai, China) and listed in Table S1. Milli-Q water (18.2 MΩ/cm) was used to prepare the buffer solution.

2.2. Analytical procedure

20 mM Tris-HCl buffer (containing 150 mM NaCl, 50 mM KCl, and 20 mM $MgCl_2$, pH 8.0) was used to prepare the DNA solution. 400 nM DNA (1) and 200 nM DNA (2) were mixed to form the DNA (1)/(2) duplex. 200 nM DNA (3), 200 DNA (4), and 400 nM DNA (5) were prehybridized to form the DNA (3)/(4)/(5) complex. Different concentrations of BPA were added to the DNA (1)/(2) duplex and incubated at 25 °C for 40 min. The DNA (3)/(4)/(5) complex and 600 nM DNA (6) were introduced and incubated for 60 min. Then, 200 nM DNA (7) and 50 nM DNA (8) were added and incubated for 45 min. The fluorescence response signals were recorded with the SpectraMax i3x (Molecular Devices, CA, USA).

In the selectivity analysis, 10 nM BPA and 100 nM control analogues, including bisphenol B (BPB), bisphenol C (BPC), ethoxylated bisphenol A (BPE), bisphenol F (BPF), diethylstibestrol (DES), and 4,4′-biphenol (4 BP) were performed in the same reaction solution.

2.3. Detection of BPA in milk samples

Milk samples were purchased from local market and filtered with 0.22 μm microfiltration membrane. The samples were diluted 10-fold using the Tris-HCl buffer for recovery assay. Different concentrations of BPA spiked in milk samples were detected by the DNA circuit biosensor. At the same time, LC–MS method was used to confirm the biosensor results.

2.4. Polyacrylamide gel electrophoresis (PAGE) experiment

5.5 mL H_2O, 3.3 mL 30% acrylamide-*N*,*N*-methylenebisacrylamide (30:1) solution, 1.0 mL 10× Tris Borate EDTA (TBE) buffer, 100 μL ammonium persulfate (APS) (10%), and 10 μL tetramethylethylenediamine (TEMED) were mixed and polymerized at room temperature for 2 h to prepare 10% PAGE gel. 3 μL reaction solution, 1 μL 6× loading buffer, and 2 μL 100× SYBR Green solution were mixed to prepare the PAGE samples. The electrophoresis analysis were carried out at 60 V for 90 min in 1× TBE buffer. The gel was scanned with the gel image analysis scanner (Bio-Rad, CA, USA).

3. Results and discussion

3.1. Sensing mechanism of the DNA circuit

The sensing mechanism of the DNA circuit for BPA detection is illustrated in Fig. 1. The aptamer sequence DNA (1) was used to recognize the target BPA. The specific interactions between DNA (1) and BPA (Chen & Zhou, 2016) lead to the release of DNA (2), which can service as an initiator sequence to activate the amplifying DNA circuit. DNA (2) first binds to the toehold domain 2* of DNA (5) to form the DNA

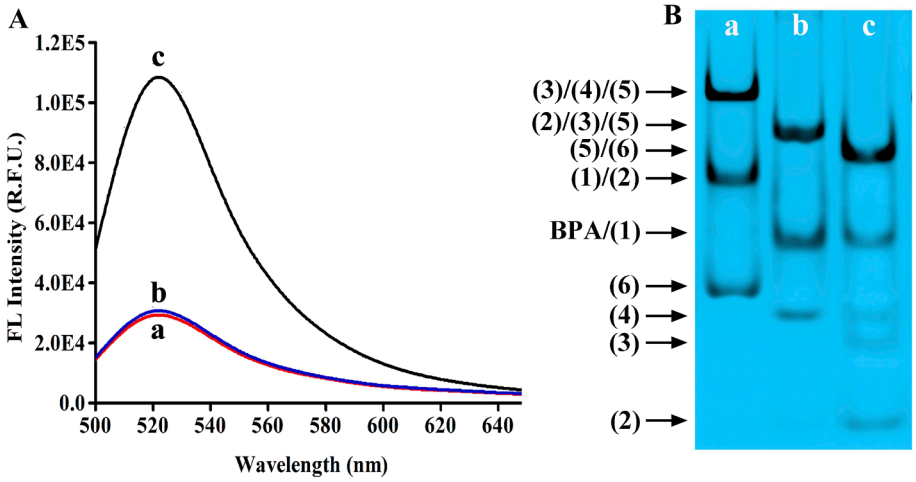

Fig. 2. (A) Fluorescence signals of the biosensor under different control conditions: (a) DNA (1)/(2) + DNA (3)/(4)/(5) + DNA (6), (b) BPA + DNA (1)/(2) + DNA (3)/(4)/(5), and (c) BPA + DNA (1)/(2) + DNA (3)/(4)/(5) + DNA (6). (B) The PAGE results at different controls: (a) DNA (1)/(2) + DNA (3)/(4)/(5) + DNA (6), (b) BPA + DNA (1)/(2) + DNA (3)/(4)/(5), and (c) BPA + DNA (1)/(2) + DNA (3)/(4)/(5) + DNA (6).

complex (DNA (2)/(3)/(4)/(5)) through toehold-mediated strand displacement (Zhang et al., 2007). The hybridization between domains 3 and 3* is too weak to keep DNA (4) stable in the complex, so DNA (4) is released and the DNA complex of DNA (2)/(3)/(5) is formed. Then, the newly exposed toehold domain 3* binds to DNA (6), resulting in another toehold-mediated strand displacement and branch migration to displace DNA (3) and DNA (2). The regenerated DNA (2) can bind to DNA (5) again, initiating the next round of the toehold-mediated strand displacement. While the DNA circuit proceeds, large amounts of DNA (4) and DNA (3) released, which can be used to construct an active Mg^{2+}-dependent DNAzyme. DNA (7) and DNA (8) are the DNAzyme strand and substrate strand of the Mg^{2+}-dependent DNAzyme, respectively. Through synergistically hybridization among DNA (3), DNA (4), DNA (7), and DNA (8), an "I" junction structure corresponded to the Mg^{2+}-dependent DNAzyme can be formed (Elbaz et al., 2010). In the presence of Mg^{2+}, the dual-labeled (FAM and Dabcyl) substrate strand will be cleaved into two segments. The separation of the fluorophore and quencher yields a high fluorescence signal, which can be used for BPA detection. In the absence of BPA, DNA (2) is blocked by DNA (1), which prevents DNA (2) to activate the DNA circuit. The number of complementary bases between DNA (7) and DNA (8) is too few to form a catalytically active DNAzyme. As a result, only background fluorescence signal can be detected. Notably, our sensing strategy is driven by toehold-mediated strand displacement and Mg^{2+}-dependent DNAzyme, without the involvement of any protein enzymes, which is beneficial to improving the sensing robustness and reducing the experimental cost.

3.2. Feasibility of the biosensor

To confirm the feasibility of the sensing system for BPA detection, the fluorescence signals were tested under different controls. As shown in Fig. 2A, in the absence of BPA, a background signal can be observed (curve a). This indicated that the trigger DNA (2) was blocked by DNA (1) and failed to activate the DNA circuit. In the presence of BPA but without DNA (6), no obvious fluorescence response can be observed (curve b). In this state, only DNA (4) could be released while DNA (3) was still inaccessible. Thus, no active DNAzyme can be formed and the signal was negligible. A significant enhancement of the fluorescence signal was achieved in the presence of both BPA and DNA (6) in the sensing system (curve c), indicating that DNA (3) and DNA (4) were released to assemble an active Mg^{2+}-dependent DNAzyme.

The PAGE experiment was also carried out to verify the design feasibility of the biosensor. As depicted in Fig. 2B, the DNA circuit without BPA yielded three bands, which represented the DNA (3)/(4)/

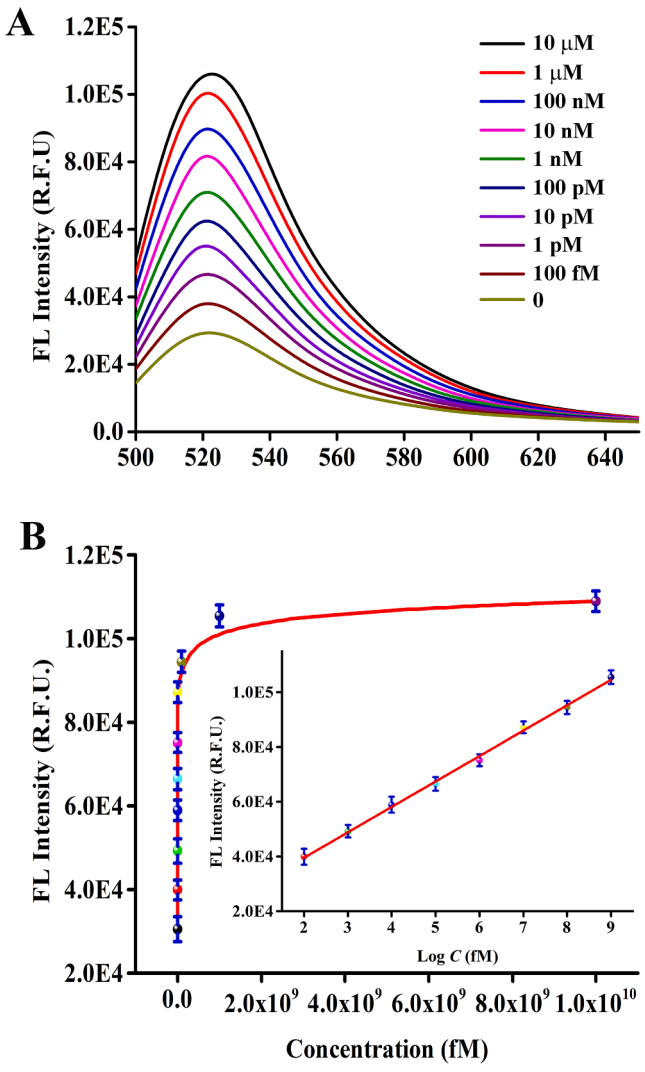

Fig. 3. (A) Fluorescence spectra of the biosensor treated with different BPA concentrations. (B) Linear relationship analysis between the fluorescence intensity at 520 nm and the BPA concentrations.

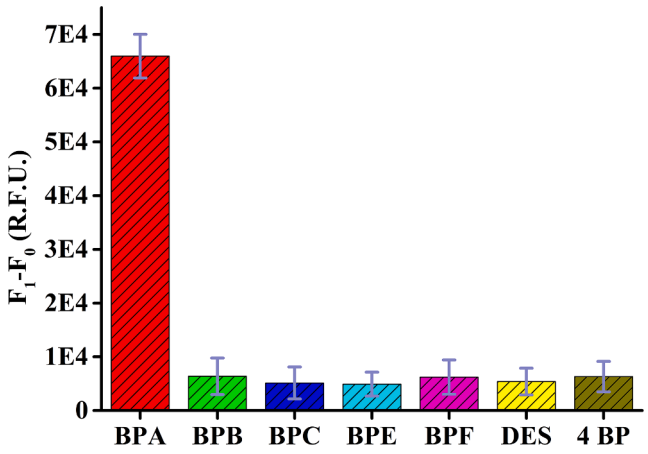

Fig. 4. Specificity investigation of the circuit for BPA against other analogues. BPA, 10 nM; other analogues, 100 nM. The error bars are standard derivation obtained from three independent experiments.

Table 1
Detection of BPA in milk samples by the proposed biosensor and LC–MS method.[a]

Samples	Spiked	Biosensor	Recovery (%)	LC–MS	Relative error (Re)[b] (%)
Milk sample 1	200 fM	207.6 fM	103.8	202.5 fM	2.5
Milk sample 2	10 pM	10.3 pM	103	9.9 pM	4.0
Milk sample 3	1 nM	0.97 nM	97	1.02 nM	−4.9
Milk sample 4	100 nM	106.4 nM	106.4	103.6 nM	2.7
Milk sample 5	1 μM	0.94 μM	94	0.98 μM	−4.1

[a] The data reported in the table represent the average of three measurements.
[b] Re: Biosensor detection *vs.* LC–MS method.

(5) complex, the DNA (1)/(2) duplex, and DNA (6), respectively (lane a). In the presence of BPA but without DNA (6), three bands corresponding to the DNA (2)/(3)/(5) intermediate, the BPA/DNA (1) complex, and DNA (4) can be observed (lane b). When DNA (6) was also present in the circuit, five bands corresponding to DNA (5)/(6) duplex, the BPA/DNA (1) complex, DNA (4), DNA (3), and DNA (2) can be observed (lane c). This results demonstrated that our designed DNA circuit can work for BPA detection based on toehold-mediated strand displacement reactions.

3.3. Analytical performance

Under the optimized assay conditions (Figs. S1–S3), the proposed DNA circuit was used to the quantitatively detect different concentrations of BPA. As displayed in Fig. 3A, the fluorescence intensities increased continuously with increasing BPA concentrations from 0 to 10 μM. From Fig. 3B, the fluorescence response showed a good linear relationship with the logarithm of BPA concentrations from 100 fM to 1 μM, and the regression equation was $F_{520} = 1.77 \times 10^5 + 9.35 \times 10^4 \lg C$ ($R^2 = 0.98$) with a detection limit of 50 fM (S/N = 3). The results showed that the sensitivity of the DNA circuit can be compared or even superior to some reported detectors for BPA detection (Table S2). This high sensitivity is attributed to the cyclic signal conversion ability of the DNA circuit.

3.4. Specificity of the sensor

To evaluate the specificity of the DNA circuit, some BPA analogues, including bisphenol B (BPB), bisphenol C (BPC), ethoxylated bisphenol A (BPE), bisphenol F (BPF), diethylstibestrol (DES), and 4,4′-biphenol (4 BP) were employed as interfering reagents for test. As depicted in Fig. 4, only the addition of BPA caused a remarkable change in the fluorescence intensity, while no significant signal changes can be observed for the controls. These results indicated a high specificity of our proposed biosensor toward the target BPA, which can be ascribed to the high affinity between BPA and the aptamer sequence.

3.5. Real sample detection

The application of the DNA circuit was evaluated by performing a recovery test in milk samples. Different BPA concentrations were spiked into milk samples and detected using the as-proposed biosensor. The analytical results were listed in Table 1. The spiked BPA concentrations were 200 fM, 10 pM, 1 nM, 100 nM, and 1 μM. Those samples were tested by the biosensor and the calculated concentrations were 207.6 fM, 10.3 pM, 0.97 nM, 106.4 nM, and 0.94 μM, respectively. The satisfactory recovery was in the range of 94%–106.4%. At the same time, LC–MS method was used to verify the biosensor results. The LC–MS detected concentrations were 202.5 fM, 9.9 pM, 1.02 nM, 103.6 nM, and 0.98 μM, respectively. Those analytical results indicated that no obvious difference existed between the DNA circuit method and the LC–MS analysis (Fig. S4), which demonstrated that our fabricated biosensor is reliable for BPA detection in real samples.

4. Conclusions

In conclusion, we have developed a fluorescent biosensor for BPA detection based on an amplifying DNA circuit and the Mg^{2+}-dependent DNAzyme. In the sensing system, the anti-BPA aptamer was used as the recognition element for BPA binding, which can improve the detection selectivity and other control analogues did not disturb the BPA biosensor. Toehold-mediated displacement reactions in the DNA circuit and continuous cleavage reaction of the Mg^{2+}-dependent DNAzyme can significantly improve detection sensitivity, with a detection limit of 50 fM. The detection linear range of this biosensor is from 100 fM to 1 μM. The assay is robust and has been applied to quantifying BPA in milk samples with satisfactory recovery. The recovery values were in the range of 94%–106.4%. Compared with the LC–MS results, the relative error of the biosensor data is less than 5%. Importantly, the whole reaction can be completed at room temperature based on DNA isothermal hybridization. No protein enzyme was involved in the reaction system, which greatly simplified experimental operations and reduced the test cost. This detection platform is versatile and can be used for the analysis of other targets by changing the aptamer sequences.

CRediT authorship contribution statement

Jiafeng Pan: Methodology, Data curation, Writing - original draft. **Zhi Liu:** Methodology, Validation. **Junhua Chen:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing - review & editing.

Declaration of Competing Interest

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128975.

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