A dual-signaling strategy for ultrasensitive detection of bisphenol A by aptamer-based electrochemical biosensor

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**Abstract**

A facile label-free aptamer-based electrochemical biosensor has been developed for sensitive detection of bisphenol A (BPA) based on a dual-signaling amplification strategy. The aptasensor was constructed by electro-deposition of gold nanoparticles (AuNPs) onto a glassy carbon electrode (GCE), where a thiolated-modified BPA aptamer was immobilized through self-assembly and then hybridized with the biotin-modified complementary DNA probe (cDNA) to form a double-stranded DNA. Upon the highly specific interaction between the target BPA and its aptamer, cDNA was released from the electrode surface and BPA was immobilized on the sensing interface. Streptavidin-modified horseradish peroxidase-functionalized gold nanoparticle (avidin-HRP-AuNP) was chosen as the nanoprobe, due to its catalytic activity to the oxidation of hydroquinone (HQ) in the presence of H₂O₂. As a result, the captured amounts of avidin-HRP-AuNP decrease with the increase of the BPA concentration and produce a series of decreasing catalytic peak currents. In addition, BPA has a redox activity and could provide an additional signal transformation. By superimposing the two signal changes, BPA was detected sensitively in a linear range from 0.001 to 1 nM with a detection limit of 0.41 pM. The aptasensor exhibited good selectivity toward BPA even in the presence of the interferents at 100-fold concentrations. This method would be readily applicable for sensitive detection of other redox analytes, merely by changing the anti-BPA aptamer/cDNA pair with a correspondent anti-target molecule aptamer and cDNA.

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1. Introduction

Bisphenol A (BPA) is one of important industrial compounds in fabrication of various consumer products, such as plastic food containers, water bottles, nursing bottles and packaging [1–3]. It can easily migrate into food, drinking water and environment and thus humans may routinely ingest trace amounts of BPA. However, BPA is a known endocrine disrupting compound, with a structure similar to that of the hormone estrogen [4,5]. Therefore, even low concentrations (ca. 100 pM) of BPA are able to bind estrogen receptors, leading to reproductive disorders, chronic diseases, diabetes and various types of cancer [6,7]. To protect human health, Canada and China banned the use of BPA in baby bottles in September 2010 and March 2011, respectively [8]. And in January 2015, the European Food Safety Authority indicated that the current Tolerable Daily Intake level for BPA is 4 μg/kg body weight/day [9]. Thus, it is urgently needed to develop fast, specific and sensitive analytical methods for determination of BPA in various specimens.

Until now, several approaches have been reported for detecting BPA, harnessing high performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry (LC/MS), gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS) [10–13]. It has been reported that these techniques are highly sensitive and could reach an ideal level for the detection of BPA. Nonetheless, all of these methods require large and expensive instrument, strict and time-consuming sample preparation steps and skilled operators, which restrict their wide applications. Hence, in order to overcome these limitations, electrochemical biosensors have proved quite promising owing to their simple, fast and cost-effective properties.

Among various electrochemical biosensors, aptamer-based ones have attracted substantial attention in numerous fields such as biochemistry, analytical chemistry, and clinic detection [14,15]. Aptamers are new type of single-stranded DNA or RNA oligonucleotides biorecognition probes, which show high selectivity to their targets with strong specificity and affinity [16,17]. Recently, several aptasensing strategies have been widely used for BPA detection, including surface-enhanced Raman spectroscopy, fluorescence, colorimetry and electrochemical method [18–21]. Notably, electrochemical aptamer sensors have attracted great attention. So far, most reported aptamer-based biosensors were based on only single signal, either the “signal-on” or “signal-off” strategies [22–25]. The “signal-off” mechanism suffered from limited signal capacity, in which only a maximum of 100% signal suppression could be attained under any experimental conditions [26,27]. In contrast, “signal-on” sensing method could achieve enormous...
signal gain as the background observed in the absence of target is pushed to zero [28,29]. Recently, to circumvent the defect of limited signal capacity of “signal-off” method and make use of the superiority of “signal-on” method, the dual-signalizing electrochemical sensor strategy has been reported [30,31]. Compared with single-signalizing method, it exhibited higher sensitivity and good selectivity.

In this paper, we developed a high selective and sensitive electrochemical aptasensor for the detection of BPA in water. Two signal indicators were employed to monitor the concentrations of BPA. Firstly, enzyme-based assays have been routinely used because they are fast, specific, easy to perform and can magnify the detection signal [32–34]. Thus an avidin-HRP-AuNP probe was prepared and used as the signal marker for the quantification of BPA, which could catalyze the oxidation of HQ in the presence of H₂O₂ to benzoquinone (BQ) and produce electrochemical signal. Additionally, it has been reported that BPA with redox activity could provide an additional signal and contributing to the signal response [22,35,36]. Taking advantages of both the HRP-catalyzed signal amplification and the direct electrochemistry of the redox target BPA, a new type of electrochemical aptasensor was developed for sensitive detection of BPA. As shown in Scheme 1, AuNPs was electrodeposited on the GCE surfaces, on which the thiolate-modified BPA aptamer was self-assembled via the Au–S bond. Then, we employed a designed complementary biotin-labeled cDNA as a detection probe to capture the avidin-HRP-AuNP nanoprobe by the specific binding of biotin-avidin. Upon the specific interaction between BPA and its aptamer, the aptamer/cDNA duplex was dissociated, and the cDNA was released from the sensing interface, which resulted in “signal-on” of BPA signal (ΔI_{BPA}) and “signal-off” of avidin-HRP-AuNP catalyzed signal (ΔI_{HRP-BQ}). Based on this protocol, sensitive and selective detection of BPA could be realized by superimposing the two signal changes (ΔI = |ΔI_{HRP-BQ}| + ΔI_{BPA}).

2. Experimental

2.1. Reagents and chemicals

Chloroauric acid (HAuCl₄·4H₂O), hydroquinone, hydrogen peroxide (H₂O₂), were obtained from Shanghai Reagent Company (Shanghai, China). 6-mercapto-1-hexanol (MCH), BPA, bisphenol B, nafion, testosterone, estradiol and 4-aminophenol were purchased from Sigma-Aldrich (St. Louis, MO). Streptavidin-modified horseradish peroxidase (avidin-HRP) and all HPLC-purified oligonucleotides were synthesized by Sangon Biological Engineering Technological (Co., Ltd. Shanghai, China). Their base sequences are listed below:

**Anti-BPA aptamer probe:** 5′-SH-(CH)₂-TTTTTTTTTCGGTGTTGTC AG GTGGGATAGCGTTCCGCGTATGGCCCAAGGCATACGCGGTTCGGCACA-3′;

**cDNA:** 5′-biotin-TACCTTCAATGTACTGTCGAAAACCCGATGCGC-3′;

(The italic bold denotes the complementary sequences.)

DNA sequences, BPA and other stock solutions were prepared in 0.1 M Tris–HCl buffer solution (pH 7.4, containing 200 mM NaCl, 25 mM KCl, 10 mM MgCl₂ and 5% ethanol). Phosphate-buffered solution (PBS, pH 7.4) was prepared by mixing the stock solutions of NaH₂PO₄ and Na₂HPO₄ and used as a working solution.

2.2. Apparatus

All electrochemical measurements were carried out on a CHI 660D electrochemical workstation (Shanghai Chenhua Apparatus Co. Shanghai, China) using a conventional three-electrode configuration with platinum wire as auxiliary electrode, saturated calomel electrode (SCE) as reference electrode and modified GCE (3 mm in diameter) as working electrode.

2.3. Synthesis of avidin-HRP-AuNP nanoprobe

First, the AuNPs were synthesized according to the previous protocol [37]. In short, 100 mL HAuCl₄·4H₂O solution (0.01%) was boiled with vigorous stirring, and then 2.5 mL trisodium citrate solution (1.0%) was quickly added. When the solution turned deep red, indicating the formation of AuNPs. Upon continued stirring and cooling down, the AuNPs colloidal solution was obtained and stored in brown glass bottles at 4 °C before use.

Then, 20 μg avidin-HRP were added to 1.0 mL colloidal AuNPs which were adjusted to pH 9.0 by 0.1 M K₂CO₃ and gently mixed at room temperature for 60 min [34]. After 30 min centrifugation at 8000 rpm and washing the soft red sediment with PBS (pH 7.4), the resulted avidin-

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Scheme 1. Schematic illustration of the fabrication of the aptasensor.

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HRP-AuNP nanoprobe was finally resuspended in 1.0 mL of pH 7.4 PBS containing 0.1% BSA and stored at 4°C prior to use.

2.4. Preparation of aptasensor

Prior to modification, the surface of the GCE was polished to a mirror-like smoothness with 0.5 and 0.05 μm alumina slurry, and then washed successively with ethanol and water. After being dried under a stream of nitrogen at room temperature, the GCE was immersed in 0.1% HAuCl₄ solution containing 0.1 M KCl to electrodeposited AuNPs with constant potential at −0.2 V for 30 s. Subsequently, 8 μL 0.5 μM BPA aptamer was cast to the AuNPs/GCE by Au-S bond for 12 h at room temperature in a 100% moisture-saturated environment. Then, the resulting electrode was further incubated with 1.0 mM MCH blocking solution for 30 min to block the possible remaining active AuNPs sites against nonspecific adsorption of the aptamer. After that, 8 μL 0.5 μM cDNA was dropped on the resulted MCH/aptamer/AuNPs/GCE electrode for 3 h to form DNA duplex. Finally, the cDNA/MCH/aptamer/AuNPs electrode was incubated with various concentrations of BPA for 3 h. The procedure of the biosensor fabrication is illustrated in Scheme 1.

2.5. Measurement procedure

The above electrodes were dipped with 8 μL avidin-HRP-AuNP solution for 60 min at room temperature and then rinsed to be ready for the electrochemical measurements. Electrochemical measurements were performed by the differential pulse voltammetry (DPV) in 0.1 M PBS (pH 7.4) containing 1.0 mM [Fe(CN)₆]³⁻/⁴⁻ and 0.1 M KCl. Cyclic voltammetry (CV) experiments were carried out in 0.1 M PBS (pH 7.4) containing 1.0 mM [Fe(CN)₆]³⁻/⁴⁻ and 0.1 M KCl at the scan rate of 100 mV s⁻¹.

3. Results and discussion

3.1. Characterization of Au nanoparticles

The morphology of the Au nanoparticles was characterized by transmission electron microscope (TEM). As displayed in Fig. 1, the Au nanoparticles uniformly dispersed with sizes of about 13 nm.

3.2. Electrochemical characterization of aptasensor

CV was employed to validate the fabrication of the aptasensor in 0.1 M PBS (pH 7.4) containing 1.0 mM [Fe(CN)₆]³⁻/⁴⁻ and 0.1 M KCl. As shown in Fig. 2, the bare GCE shows a well-defined reversible redox peak (curve a). After deposition of AuNPs on the GCE surface, an obvious increases of peak current is observed (curve b), suggesting good electrical conductivity of the AuNPs membrane. When the BPA aptamer was immobilized on the electrode, the redox current of [Fe(CN)₆]³⁻/⁴⁻ dramatically decreased (curve c), presumably due to the electrostatic repulsion between the negatively charged thiolate-aptamer and [Fe(CN)₆]³⁻/⁴⁻, this decrease in redox current confirmed the successful immobilization of the aptamer onto the electrode surface. Afterwards, to minimize nonspecific adsorption, the fabricated electrode was treated with MCH. The coverage of MCH minimized the exposed surface area of the AuNPs, thereby decreasing the redox current of [Fe(CN)₆]³⁻/⁴⁻ (curve d). The peak current further decreases after the MCH/aptamer/AuNPs/GCE hybridized with cDNA (curve e), attributing to the formation of double-stranded DNA which augments the negatively charges of the electrode surface. Similarly, after the aptasensor was incubated in BPA, a dramatic decrease in current relative to curve d was observed (curve f), because the complementary pairing of BPA and aptamer could impede the electron transfer tunnel.

3.3. Dual-signaling strategy of aptasensor response

The feasibility of the designed dual-signaling strategy aptasensor for BPA detection was investigated by DPV in a series of control experiments, as shown in Fig. 3. After incubation with 8 μL avidin-HRP-AuNP nanoprobes, no redox current was observed for the nanoprobe on MCH/aptamer/AuNPs/GCE (curve a), suggesting that the electrode could not capture the nanoprobe. However, a strong catalyzed peak current of HRP appeared at about −0.10 V for the cDNA/MCH/aptamer/AuNPs/GCE electrode (curve b) due to the specific integration between the avidin-HRP-AuNP and biotin-modified cDNA. After the modified electrode was incubated with 0.1 nM target BPA. The response signal greatly decreases (curve c), which confirms the successful interaction between BPA and its aptamer, leading to the dissociation of cDNA/aptamer duplex and release of the cDNA from electrode surface, due to the higher affinity constant between BPA and its aptamer than that of the related aptamer/cDNA duplex. Furthermore, another redox current appears at about 0.5 V due to the inherent redox activity of BPA. Based on the above results, it is no doubt that the developed aptasensor can be employed in BPA detection based on the dual-signaling protocol.

Fig. 1. TEM image of Au nanoparticles.

Fig. 2. CV of different modified electrodes in 0.1 M PBS (pH 7.4) containing 1.0 mM [Fe(CN)₆]³⁻/⁴⁻ and 0.1 M KCl: (a) bare GCE, (b) AuNPs/GCE, (c) aptamer/AuNPs/GCE, (d) MCH/aptamer/AuNPs/GCE, (e) cDNA/MCH/aptamer/AuNPs/GCE, (f) BPA/cDNA/MCH/aptamer/AuNPs/GCE.
1.0 mM HQ.


BQ$^+$H$_2$O$_2$, HQ was oxidized to BQ, and BQ could engage in electron exchange on the electrode by the specific interaction between avidin and biotin-labeled cDNA. Through the HRP-catalyzed reaction in the presence of the electrode surface by DPV. The reaction mechanism of the catalytic process can be expressed as [38]:

\[
H_2O_2 + \text{HRP}_{\text{red}} \rightarrow \text{HRP}_{\text{ox}} + H_2O \tag{1}
\]

\[
\text{HRP}_{\text{ox}} + HQ \rightarrow \text{BQ} + \text{HRP}_{\text{red}} \tag{2}
\]

\[
\text{BQ} + 2H^+ + 2e^- \rightarrow \text{HQ} \tag{3}
\]

In order to verify the amplification effect HRP, the avidin-HRP-AuNP/cDNA/MCH/aptamer/AuNPs/GCE electrode was incubated in different experimental conditions. As seen in Fig. 4A, no redox response is observed in pH 7.4 PBS (curve a) or with 0.6 mM H$_2$O$_2$ (curve b). With the addition of 1.0 mM HQ only, a reduction peak of HQ appears (curve c). However, when both 1.0 mM HQ and 0.6 mM H$_2$O$_2$ are added, a significant reduction peak current is observed (curve d). So the mixture of 1.0 mM HQ and 0.6 mM H$_2$O$_2$ was employed in the subsequent experiments for catalytic substrates.

In addition, using AuNPs as matrix in the HRP nanoprobe may enlarge the amplification effect of enzymatic response. We compared the DPV peak current of avidin-HRP/cDNA/MCH/aptamer/AuNPs/GCE (curve a) and avidin-HRP-AuNP/cDNA/MCH/aptamer/AuNPs/GCE (curve b) in 0.1 M PBS (pH 7.4) containing 0.6 mM H$_2$O$_2$ and 1.0 mM HQ. In the Fig. 4B, it is noted that the curve b is about 2 times higher than curve a, in which the AuNPs can load high amounts of HRP and increase the number of HRP on the electrode surface. So in the whole experiment, we chose avidin-HRP-AuNPs as the nanoprobe.

3.4. Amplification effect of avidin-HRP-AuNP

HRP is chosen as the signal amplification indicator because of its high sensitivity and substrate specificity. In this work, streptavidin-modified HRP was designed as the signal marker, and it can be bounded by the electrode through the specific interaction between avidin and biotin-labeled cDNA. Through the HRP-catalyzed reaction in the presence of the electrode and turn back into HQ itself. Therefore, HQ recycles in this system causing the amplification of the reduction current. The detection was based on the reduction of BQ, which generated on the electrode surface by DPV. The reaction mechanism of the catalytic process can be expressed as [38]:

3.5. Optimization of experimental conditions

The effect of deposition time of AuNPs was tested in the range of 10–50 s. As seen in the Fig. 5A, the highest current response of [Fe(CN)$_6$]$^{3-/4-}$ is realized at a deposition time of 30 s. So 30 s of deposition time is used for AuNPs preparation on the electrode surface.

To obtain the high sensitivity, we incubated different concentrations of aptamer on the AuNPs/GCE surface (Fig. 5B). The [Fe(CN)$_6$]$^{3-/4-}$ redox signal decreased as the concentration of aptamer increasing from 0.1 to 1.0 μM, due to the electrostatic repulsion between the negatively charged aptamer and [Fe(CN)$_6$]$^{3-/4-}$. The lowest of current is realized at the concentration of 0.5 μM and the signal keeps almost unchanged as the concentration exceeds 0.5 μM, indicating the near-saturation occurred. Hence, 0.5 μM is chosen as the optimized concentration of aptamer.

The effect of different amounts of cDNA from 0.1 to 1.0 μM were also evaluated. As shown in Fig. 5C, the CV response further decreases with the increase of the cDNA concentration, because of the formation of DNA duplex increases the negatively charges on the electrode surface. The signal almost stays stable when it exceeds 0.5 μM. Therefore, 0.5 μM cDNA was employed in the subsequent experiments for binding with aptamer.

The effect of the hybridization time between aptamer and cDNA on the CV response of [Fe(CN)$_6$]$^{3-/4-}$ was also evaluated. As shown in Fig. 5D, the peak current obviously decreases with the increase of hybridization time from 0.5 to 5 h, and almost remains stable after 3 h, suggesting that the hybridization is completed. Thus, 3 h is selected as the optimum hybridization time for cDNA.

3.6. Analytical performance of designed biosensor

Under the optimized conditions, the prepared aptasensor was employed to detect various concentrations of BPA in 0.1 M PBS

![Fig. 3. DPV responses of different electrodes in PBS (pH 7.4) containing 1.0 mM HQ and 0.6 mM H$_2$O$_2$ after incubated with 8 μL avidin-HRP-AuNP nanoprobes: (a) MCH/aptamer/AuNPs/GCE, (b) cDNA/MCH/aptamer/AuNPs/GCE, (c) BPA/cDNA/MCH/aptamer/AuNPs/GCE. The concentration of BPA is 0.1 nM.](image)

![Fig. 4. (A) DPVs of avidin-HRP-AuNP/cDNA/MCH/aptamer/AuNPs/GCE electrode in 0.1 M PBS (pH 7.4) containing (a) 0, (b) 0.6 mM H$_2$O$_2$, (c) 1.0 mM HQ, (d) the mixture of 0.6 mM H$_2$O$_2$ and 1.0 mM HQ. (B) DPVs of (a) avidin-HRP-cDNA/MCH/aptamer/AuNPs/GCE, (b) avidin-HRP-AuNP/cDNA/MCH/aptamer/AuNPs/GCE in 0.1 M PBS (pH 7.4) containing 0.6 mM H$_2$O$_2$ and 1.0 mM HQ.](image)

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(pH 7.4) containing 1.0 mM HQ and 0.6 mM H2O2. As shown in Fig. 6A, the DPV response of avidin-HRP-AuNPs catalyzed signal decreases while BPA redox signal increases with the increasing concentration of BPA. Fig. 6B shows the relationship between the peak currents of HRP

Fig. 5. Optimization of experimental parameters by CV peak current in 1.0 mM [Fe(CN)6]3−/4− solution containing 0.1 M KCl: (A) deposition time of AuNPs, (B) aptamer concentration, (C) cDNA concentration, (D) hybridization time between aptamer and cDNA.

Fig. 6. (A) DPV curves for the detection of different concentrations of BPA (from a to i): 0, 0.003, 0.008, 0.01, 0.1, 1.0 nM. Relationship between the (B) ΔHRP-BQ, (C) ΔBPA, (D) ΔHRP-BQ + ΔBPA and the negatively logarithm concentration of BPA.

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catalyzed signal and the negatively logarithm of BPA concentration, it could be found that the peak current is negatively related to the negatively logarithm of BPA concentration in the range from 0.001 nM to 1 nM. The regression equation is $\Delta \text{I}_{\text{bioelectrode}} = 34.90 + 2.83 \log_{10} C_{\text{BPA}}$ ($R^2 = 0.992$), and the limit of detection is calculated as 0.55 pM based on the three times of signal-to-noise value. According to the same procedure, the detection limit is about 0.93 pM based on individual BPA signal with the regression equation $\Delta \text{I}_{\text{bioelectrode}} = 44.73 + 3.48 \log_{10} C_{\text{BPA}}$ ($R^2 = 0.992$). However, by superimposing the two oxidation peak current changes, the regression equation is $\Delta \text{I}_{\text{bioelectrode}} = 79.11 + 6.27 \log_{10} C_{\text{BPA}}$ ($R^2 = 0.994$) and the detection limit is calculated to -0.41 pM. It is worthy to note that the limit of detection value by this dual-signaling assay is much lower than the single-signaling assay. In addition, compared with other previously reported electrochemical BPA biosensors, the developed strategy shows more sensitivity for the detection of BPA [17,24,25,35,39,40].

To investigate the specificity of the as-prepared biosensor, the influences of some potential interferents on the determination of BPA were investigated. Testosterone, nafion, 4-aminophenol, estradiol and bisphenol B were chosen as the possible interfering substances of target BPA. As shown in Fig. 7, the developed aptasensor exhibited a strong response to BPA compared with these interferents, despite that their concentrations were 100 times than the target BPA concentrations. Additionally, the response signals in the solutions containing BPA only and the mixture are almost the same. These results indicate that the proposed aptasensor has good specificity and selectivity toward BPA detection due to the specific recognition of BPA aptamer.

The sensors exhibited satisfactory long-term stability and reproducibility. Five prepared electrodes incubated with the same BPA concentration (0.01 nM) were used to investigate the reproducibility of the aptasensor under the same conditions. The signal responses of the five successive measurements by DPV were determined and a relative standard deviation (RSD) of 4.5% was obtained, which suggests an acceptable reproducibility of the aptasensor.

3.7. Practical application

The reliability of this sensor in practical applications was evaluated by performing a recovery test in tap water. The water samples were diluted two times with 0.1 M PBS, and then spiked with different concentrations of BPA. The results showed that the recoveries are between 95% and 105%, and the RSDs are in the range of 2.3–5.5%. These satisfactory results indicated that this aptasensor was feasible for the analysis of real samples.

4. Conclusion

A novel dual-signaling electrochemical aptasensor was developed for sensitive and selective detection of BPA, by coupling the signal amplification of avidin-HRP-AuNP nanoprobe with the inherent redox activity of target BPA. The highly specific interaction between BPA and its aptamer led to the release of cDNA from the electrode surface and decrease the binding amounts of avidin-HRP-AuNP nanoparticles, which resulted to the decrease of the catalyzed peak current of avidin-HRP-AuNP and the increase of oxidation peak current of BPA. By superimposing the two signal changes, BPA was detected sensitively in a linear range from 0.001 to 1 nM with the detection limit of 0.41 pM, which is more sensitive than single-signaling assays. This strategy also exhibited good specificity, stability and reproducibility. Importantly, merely by replacing the anti-BPA aptamer/cDNA pair with a suitable anti-target molecule aptamer and cDNA, this method would be readily applicable for the detection of other redox targets, and be promising for the analysis of real samples with excellent recovery.

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References


Fig. 7. Specificity evaluation of the proposed aptasensor for 0.01 nM BPA against 1.0 nM interferents: (a) testosterone, (b) nafion, (c) estradiol, (d) 4-aminophenol, (e) bisphenol B, (f) bisphenol A and (g) BPA in the mixture is 0.01 nM.

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