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ORIGINAL PAPER

DNA biosensor based on a glassy carbon electrode modified with electropolymerized Eriochrome Black T

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Abstract
We report on an electrochemical DNA biosensor consisting of a glassy carbon electrode modified with a film of electropolymerized Eriochrome Black T (pEBT) that serves as a functional platform for the immobilization of probe DNA. pEBT was deposited via cyclic voltammetry, and the amino-modified DNA capture probe was covalently linked to the surface via a sulfanilamide coupling reaction. The single step of the assembly process was monitored by atomic force microscopy and electrochemistry. The surface density of DNA probe on the biosensor interface was calculated to be 1.7×10^{-10} mol cm^{-2} using methylene blue as an electroactive probe. Hybridization experiments showed the peak currents of methylene blue to decrease with increasing concentration of complementary sequence in the range from 5.0 f. to 5.0 pM. The detection limit is as low as 0.11 fM. Selectivity studies showed that the biosensor can discriminate a fully complementary sequence from a single-base mismatch, three-base mismatch, and a fully non-complementary sequence. The biosensor displays good stability and can be regenerated due to the beneficial effects of electropolymerization and covalent immobilization of probe DNA.

Keywords DNA biosensor · Eriochrome black T · Electropolymerization · Methylene blue

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Introduction

In recent years, hybridization reaction-based electrochemical sensing method attracts much attention for DNA analysis, since it has considerable advantages of low-cost, simplicity, miniaturization and high sensitivity [1, 2]. In a typical electrochemical sensing scheme, the target DNA fragment is detected by monitoring the hybridization reaction with the single-stranded probe DNA that anchored on a support platform [1, 2]. In such a scheme, the immobilization of probe DNA on the solid surface is a critical step because it has great influence on the analytical performance of the biosensor such as hybridization efficiency, stability and sensitivity [3, 4]. To date, many methods such as physical absorption [5], electrostatic interaction [6] and covalent coupling [7, 8] have been exploited. Among these, the covalent coupling method receives great attention since it not only provides a stable detection layer preventing probe DNA desorption from the electrode surface, but also gives a favorable probe DNA orientation for hybridization reaction [7, 8].

In order to improve the immobilization effect of probe DNA on the electrode surface, many new materials such as nano-sized particle and polymers have been modified on the electrode surface as DNA immobilization platform [9–13]. The polymers films modified electrodes attract extensive interest in the field of DNA biosensor fabrication due to its high homogeneity, strong adherence to electrode surface and excellent chemical stability [12, 13]. Electropolymerization of organic molecules is a favorable approach to prepare polymer-modified electrode because the thickness, permeation, character and functionality of the film can be well controlled by this method [14].

Eriochrome black T (EBT) is a classical azo sulfonic acid dye, which has been often used as the spectroscopic probe for the determination of metal ion and biological molecules [15, 16]. In recent years, the electropolymerization and the biosensing application of its polymer also attract much

attention. For example, Yao et al. [17] recently electrochemically polymerized eriochrome black T (pEBT) on a glassy carbon electrode (GCE) for the simultaneous detection of dopamine, ascorbic acid and uric acid. The results showed that the redox peak of the three analytes could be well distinguished and their peak intensities were conspicuously enhanced by the pEBT film. Liu et al. [18] have also used Au-nanoparticle/pEBT composite film as a sensing platform for the detection of *L*-cysteine and *L*-tyrosine, and the high sensitivity with the high selectivity were obtained for the two species. However, the application of pEBT as a supporting material for the graft of biomolecules has not been reported yet.

From the previous work by Geng et al. [19], it has been known that the functional groups such as –OH, SO_3^- are still remained on the polymer film of pEBT, which therefore can be considered to react with some biological molecules such as DNA and proteins to construct novel biosensors. We describe here a novel DNA biosensor that was fabricated based on the sulfamide coupling reaction between the free sulfonic groups on pEBT and the modified amino groups on probe DNA. Firstly, the pEBT film was prepared through the classical electrochemical method on a glassy carbon electrode, and then the sulfonic groups on the electropolymerized EBT layer was transferred to the active sulfonyl chloride by PCl_5, followed by the grafting with the probe DNA through the sulfamide coupling reaction. Characterization experiments indicated that a high DNA loading density could be achieved by this approach. DNA hybridization assays further showed that a wide concentration range from 5.0 f. to 5.0 pM with a low detection limit of 0.11 f. could be detected for the complementary target DNA. Good selectivity, regeneration ability and reproducibility were also achieved for the developed biosensor, which opens new opportunity for the biosensing application of the polymer based on the sulfonic acid dyes.

Experimental

Reagents and apparatus

Eriochrome black T (EBT) and methylene blue (MB) were purchased from Shanghai Chemical Reagents Company (China, http://shhuagongshiji.ebdoor.com/), and used without further purification. Phosphorus pentachloride (PCl_5) was obtained from Aladdin Reagent Company (China, http://www.aladdin-reagent.com/). Phosphate buffer solution was prepared by mixing 20 mM NaCl and 25 mM NaH_2PO_4-Na_2HPO_4. Ethylenediamine tetraacetate (EDTA) and Tris (hydroxymethyl) aminomethane (Tris) were provided by Xilong Chemical Company (China, http://xlreagent.cn.1688.com). All the other chemicals were of analytical reagent grade and deionized water was used throughout this work.

The 18-base synthetic oligonucleotides from cauliflower mosaic virus (CaMV) 35S promoter gene were purchased from Shanghai Sangon Bioengineering Limited Company (China, http://www.sangon.com/). Their base sequences were as follows:

- Amino modified probe sequence (S1):5′-NH_2-$(CH_2)_6$-TCT TTG GGA CCA CTG TCG-3′
- Complementary sequence (S2): 5′-CGA CAG TGG TCC CAA AGA-3′
- One-base mismatch sequence (S3): 5′-CGA CAG TGG TCC CAA **C**GA-3′
- Three-base mismatch sequence (S4): 5′-CGA CA**A** TGG **C**CC CAA **C**GA-3′
- Non-complementary sequence (S5): 5′-GCA TCG AGC GAG CAC GTA-3′

Stock solutions (10 μM) of all oligonucleotides were prepared with TE buffer solution (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0) and kept frozen.

The morphological characterization of the electrode before and after electropolymerization with EBT was carried out with atomic force microscopy CSPM5500 (China, http://benyuan.instrument.com.cn/). The electrochemical experiments were measured on a CHI 650C electrochemical analyzer (China, http://www.chinstr.com/) with the conventional three-electrode system consisted of bare GCE (Φ=2 mm) or modified GCE as working electrode, Ag/AgCl/(3.0 M) KCl as reference electrode and platinum wire as auxiliary electrode.

Procedures

Electropolymerization of Eriochrome Black T on glassy carbon electrode

Prior to use, the bare GCE was polished to a mirror-like surface with 1.0 μm, 0.3 μm and 0.05 μm alumina slurry, and then sonicated in acetone, ethanol and water for 5 min in turn. After that, the cleaned GCE was successively scanned in 10 mM EBT solution in the potential range from −0.4 V to + 1.5 V with the scan rate of 20 mV s^{-1}. When the voltammetric curves were stable, the scanning was terminated and the modified electrode was rinsed with water to remove the physically absorbed EBT molecules. Thus, the pEBT modified GCE (pEBT/GCE) was obtained.

Probe DNA immobilization and its hybridization

The covalent immobilization of probe DNA on pEBT/GCE was achieved by a facile sulfanilamide coupling reaction [20]. In brief, the pEBT/GCE was first immersed in 2 mL acetone containing 40 mM PCl_5 for 20 min to convert the sulfonic

groups on pEBT into the active sulfonyl chloride. Then 10 μL of 10 μM S1 solution was cast onto the electrode surface for sulfamide reaction. After dryness, the modified electrode was washed with TE buffer to remove the physically absorbed DNA, and thus the electrode with the immobilized probe DNA was obtained, which was denoted as S1/pEBT/GCE.

The hybridization reaction was performed by incubating S1/pEBT/GCE in TE buffer solution containing the desired concentration of target DNA (S2) for 45 min at 42 °C. Afterwards, the electrode was washed with TE buffer to remove the unhybridized DNA. The obtained hybridized electrode was denoted as S2-S1/pEBT/GCE. The hybridization of the S1/pEBT/GCE with the one-base mismatched DNA (S3), the three-base mismatched DNA (S4) and the non-complementary DNA (S5) were performed through the similar procedures, and the obtained electrodes were denoted as S3-S1/pEBT/GCE, S4-S1/pEBT/GCE and S5-S1/pEBT/GCE, respectively.

Electrochemical detection

Electrochemical characterization of the step-by-step assembly process of the biosensor was carried out in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The scan rate in CV was 100 mV s^{-1} and the potential window was between −0.2 V and +0.6 V. The EIS was collected at a potential of +0.172 V in the frequency range from 0.5 to 10 kHz with the voltage amplitude of 5 mV.

The hybridization detection of the biosensor was performed by using MB as the redox indicator according to the following procedures. Firstly, the different modified electrodes were incubated into 0.1 mM phosphate buffer solution (pH 7.0) containing 10 μM MB for 30 min under open-circuit. Then the electrodes were rinsed with phosphate buffer solution to remove the nonspecifically bound MB. The electrochemical behaviors of MB that have specifically bound on the biosensor were operated in phosphate buffer solution without MB via CV and differential pulse voltammetry (DPV). CV was scanned between −0.7 V and +0.6 V. DPV was scanned from −0.7 V to 0 V with pulse amplitude of 50 mV, pulse width of 50 ms, pulse period of 200 ms and increasing potential of 4 mV. The main process for the fabrication and detection strategy of the electrochemical DNA biosensor is illustrated in Fig. 1.

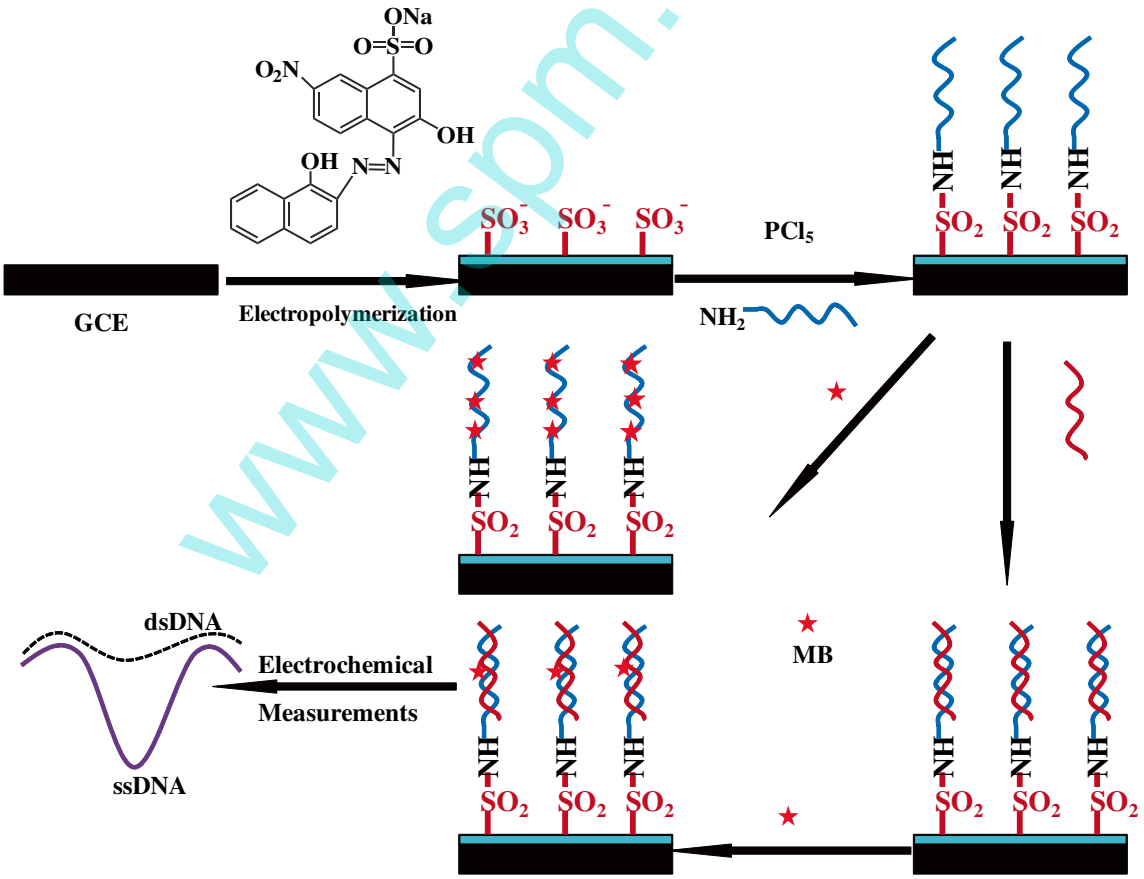

Fig. 1 Diagram of fabrication and detection process of the electrochemical DNA biosensor

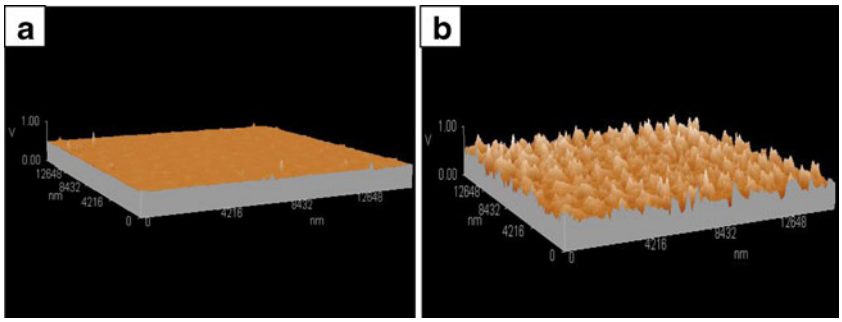

Fig. 2 AFM images of GCE before (**a**) and after electropolymerization with EBT (**b**)

Results and discussion

Electropolymerization of eriochrome black T on glassy carbon electrode

Electropolymerization of EBT on GCE was achieved by successive scanning in 10 mM EBT in the potential range from −0.4 V to +1.5 V, and the obtained results are showed in the Fig. S1 of the Electronic Supplementary Material (ESM). From the figure, it can be observed that two irreversible oxidation peaks appeared at +0.08 V (a) and +0.78 V (b) respectively, at the first cycle. According to the literature of [21], the first peak can be ascribed to the oxidation process of the phenolic hydroxyl to the benzoquinone, and the second one was the azo bond to the diimine structure. With multicycle scanning, both of the two peaks were decreased, which indicated that the GCE surface has been continuously coated by the electropolymerized EBT film. After the electrodeposition reaction was finished, the color of the electrode surface was changed from black to gloss black, further suggesting that pEBT has been formed on the surface of the bare GCE.

AFM was also applied to testify the successful modification of pEBT on GCE. The AFM images of GCE before and after electropolymerization with EBT are showed in Fig. 2. It can be seen that the bare GCE shows a smooth surface (Fig. 2a), but on pEBT/GCE, some forest-like peaks are observed (Fig. 2b), further showing the success of the electropolymerization of EBT molecules on GCE.

Electrochemical characterization of electropolymerized eriochrome black T modified glassy carbon electrode

The electrochemical behaviors of the modified electrode were characterized by CV (Fig. 3A) and EIS (Fig. 3B) using $[Fe(CN)_6]^{3-/4-}$ ions as the redox probe. It was observed that a pair of well-defined redox peaks corresponding to the electrochemical response of $[Fe(CN)_6]^{3-/4-}$ couple was observed on bare GCE (curve a). When the GCE was electropolymerized with EBT, the redox peaks of $[Fe(CN)_6]^{3-/4-}$ couple decreased markedly with the increase of the peak-to-peak separation (curve b), which suggested that the electron transfer kinetic of the $[Fe(CN)_6]^{3-/4-}$ probe was hindered by the modified film of EBT. This could be ascribed to the electrostatic repulsion of the negatively charged sulfonic acid groups on EBT towards the negative $[Fe(CN)_6]^{3-/4-}$ ions [20]. EIS is also a sensitive and frequently used method to probe the interface properties of the modified electrodes. Commonly, the shape of a typical Nyquist diagram is consisted of an oblique line at the low frequencies region that corresponds to the diffusion process and a semicircle portion at high frequencies region corresponding to the electron transfer limiting process. The electron transfer resistance (R_{et}) value is often directly obtained by

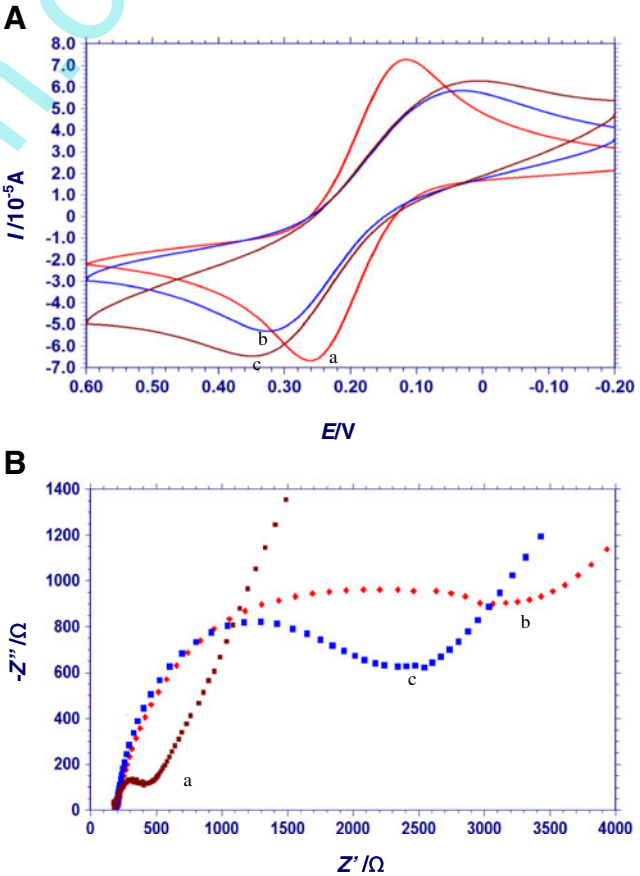

Fig. 3 CVs (**A**) and Nyquist diagrams (**B**) of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution with 0.1 M KCl on bare GCE (**a**), pEBT/GCE (**b**) and S1/pEBT/GCE (**c**)

measuring the diameter of the semicircle portion. From the EIS results, it was observed that the bare GCE showed a small semicircle with the R_{et} value of 300 Ω (curve a), indicating low electron transfer resistance on the bare GCE surface. After electropolymerization with EBT, the R_{et} value was dramatically increased to 3000 Ω (curve b), proving that the EBT had been grafted on the bare GCE, and the approaching of $[Fe(CN)_6]^{3-/4-}$ to the electrode surface was blocked. Furthermore, according to the difference value of R_{et} (ΔR_{et}) between on pEBT/GCE and bare GCE, the surface coverage (θ) of EBT on GCE surface was calculated by the following equation [22]:

$$\theta = 1 - R^{bare}{}_{et} \Big/ R^{pEBT}{}_{et}$$

Where $R^{\text{bare}}{}_{\text{et}}$ and $R^{\text{pEBT}}{}_{\text{et}}$ are the R_{et} value determined on bare GCE and pEBT/GCE, respectively. Therefore the value of θ was calculated to be 90 %, which demonstrated that most surface of the bare GCE has been covered by pEBT molecules.

Covalent immobilization of probe DNA on electropolymerized eriochrome black T modified glassy carbon electrode

The covalent immobilization of probe DNA of S1 on the surface of pEBT/GCE was also evaluated by the CV and EIS using $[Fe(CN)_6]^{3-/4-}$ as probe, and the results were depicted as curve c in Fig. 3. It was obtained that after grafting with S1, the redox peaks of $[Fe(CN)_6]^{3-/4-}$ showed enhancement in some extent in comparison with the case without immobilization of S1, which suggested that the electrochemical response of $[Fe(CN)_6]^{3-/4-}$ was changed better when pEBT/GCE was grafted with S1, which was in good agreement with that in EIS measurement, i.e., the semicircle diameter corresponding to the electron transfer resistance was decreased from 3000 Ω to 2500 Ω after reaction with S1. These changes might be related to the variation of surface status of pEBT/GCE upon immobilization with DNA probe, namely, when the GCE was electropolymerized with EBT, the electrode surface was directly coated with a layer of dense and crowed negatively charged sulfonic groups, which significantly prevented the approaching of the $[Fe(CN)_6]^{3-/4-}$ ions, resulting in inferior CV response of $[Fe(CN)_6]^{3-/4-}$ as well as large R_{et} value. However once the negative sulfonic groups were covalently coupled with the amino groups of S1, an electrically neutral sulfamide bond was formed on the electrode surface, which reduced the negative-charge density of electrode surface. Therefore, the redox molecules of $[Fe(CN)_6]^{3-/4-}$ in solution could feasibly approach the surface of S1/pEBT/GCE, resulting in a decreased electron-transfer resistance. These phenomena also proved that the probe DNA has been immobilized on the surface of pEBT/GCE via a sulfanilamide condensation reaction.

Electrochemical behaviors of methylene blue on S1/pEBT/GCE

It has been well known that the phenazine dye of MB has good electrochemical property and can specially interact with the free guanine residues of single-standed DNA [23], thereby producing the larger electrochemical response on unhybridized electrode surface than the hybridized one. Based on this, the dye is often utilized as an electrochemical probe to monitor the hybridization event in DNA biosensor [7, 20]. The MB was also applied as a hybridization indicator to investigate the immobilization and hybridization performance of the developed DNA biosensor. The CVs of MB that pre-accumulated on S1/pEBT/GCE at different scan rates in blank phosphate buffer solution was showed in Fig. S2 of ESM. It could be found that a pair of well defined redox peaks was observed on S1/pEBT/GCE, showing that MB underwent excellent electrochemical behaviors on the biosensor. The oxidation peak currents (I_p) presented an excellent linear relationship with the scan rate in the range from 0.02 V s^{-1} to 0.5 V s^{-1}, $I_p/\mu A = 23.141v/(V\ s^{-1}) + 1.708$ ($r = 0.998$) (inset of Fig. S2), which indicated that the MB molecules had been bound with S1 on the electrode surface.

Additionally, according to the method reported in literature [24], the surface densities of the DNA probes on the modified electrodes were evaluated. The brief principle of this method is firstly to calculate the molar quantity of MB (N) on S1/pEBT/GCE from the cyclic voltammetry according to the equation: $N = Q/(n e N_A)$, where Q represents the electric charge quantity during MB reduction process; n (=2) is the transfer number of electrons in the reaction; e ($=1.6\times10^{-19}$ C) is the electric charge quantity of one electron; N_A ($=6.02\times10^{23}$ mol^{-1}) represents Avogadro's number. In this experiment, the electric charge quantity of MB was measured to be 4.8×10^{-6} C from the CV measurement. Therefore, the N value of 2.5×10^{-11} mol was yielded. In addition, because one MB molecule bound with one guanine residue, and each DNA chain used contained five guanine bases, therefore, the surface density of the DNA probes was calculated to be 5.0×10^{-12} mol based on the stoichiometric ratio of n_{MB}: n_{DNA}= 5:1. The apparent area of GCE used in this experiment was 0.03 cm^2, and so the surface density of the probe DNA on the S1/pEBT/GCE was estimated to be 1.7×10^{-10} mol cm^{-2}, which was in the same order of that on the other immobilization platforms such as CeO_2–SWNTs–$BMIMPF_6$/GCE (5.4×10^{-10} mol cm^{-2}) [24], PANI-MWNTs/CHIT/CPE (4.2×10^{-10} mol cm^{-2}) [25], showing that the electropolymerization film of pEBT had the comparable effect as the nanomaterials for DNA immobilization.

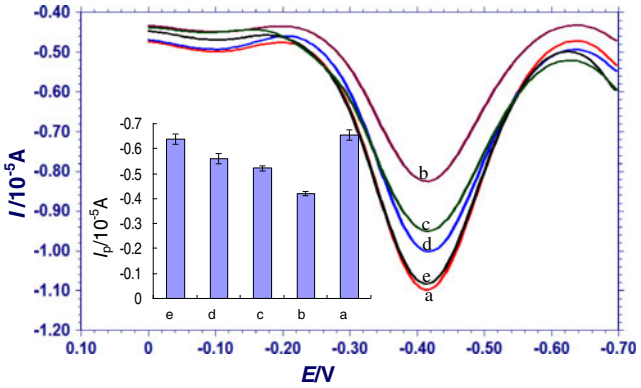

Fig. 4 DPVs (main panel) and the corresponding histogram (inset) of the peak current values 10 μM MB recorded on S1/pEBT/GCE (**a**), S2–S1/pEBT/GCE (**b**), S3–S1/pEBT/GCE (**c**), S4–S1/pEBT/GCE (**d**) and S5–S1/pEBT/GCE (**e**). The concentrations for all the hybridized sequences are 5.0×10^{-12} M

Analytical performance of the biosensor

The hybridization selectivity was first investigated to study the analytical performance of the DNA biosensor. Figure 4 shows the DPVs and the corresponding histogram (inset) of the oxidation peak currents of MB on S1/pEBT/GCE before (curve a) and after hybridization with the complementary sequence of S2 (curve b), the one-base mismatched sequence of S3 (curve c), the three-base mismatched sequence of S4 (curve d) and the non-complementary sequence of S5 (curve e). It was clearly observed that MB showed the largest peak on S1/pEBT/GCE, and the response was very similar to that on S5-S1/pEBT/GCE, indicating that no hybridization was happened between S5 and S1 due to the sequence mismatching. After hybridization with the complementary sequence of S2, the peak current of MB was decreased significantly, suggesting that the interaction of MB with guanine residues was shielded, owing to the formation of perfect double helix structure between S1 and S2. Moreover, the high selectivity of the biosensor was assessed by detecting the target sequences with one base mismatched and three base mismatched sequences. It was found that the current intensities on these two sequences hybridized electrodes are fallen in between S1/pEBT/GCE and S2–S1/pEBT/GCE, showing that only partial hybridization with S1 was accomplished for these two analytes. Additionally, the peak current on S4–S1/pEBT/GCE was somewhat larger than on S3–S1/pEBT/GCE, suggesting that the biosensor could also distinguish the target DNA with different mismatching degrees.

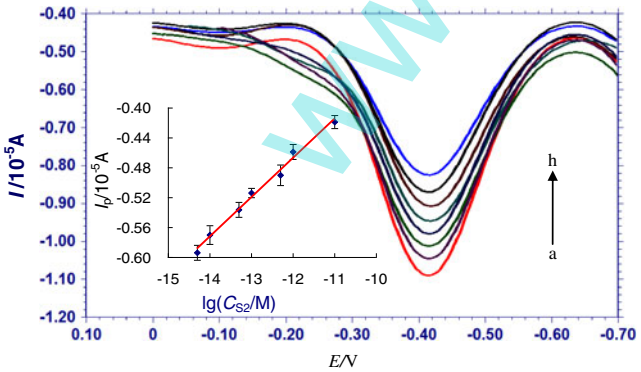

Fig. 5 DPVs of 10 μM MB accumulated on S1/pEBT/GCE before (**a**) and after hybridization with 5.0×10^{-15} M (**b**), 1.0×10^{-14} M (**c**), 5.0×10^{-14} M (**d**), 1.0×10^{-13} M (**e**), 5.0×10^{-13} M (**f**), 1.0×10^{-12} M (**g**), 5.0×10^{-12} M (**h**). Inset shows the plot of the oxidation peak currents versus the logarithm values of S2

The sensitivity of the electrochemical biosensor for the complementary target DNA was investigated by varying the complementary sequence concentrations. Figure 5 shows the DPVs of MB on S1/pEBT/GCE before and after hybridization with different concentrations of S2. It could be seen that with the increase of the concentrations of S2 (C_{S2}), the oxidation peaks of MB decreased gradually, showing that more and more duplex DNA were formed on the biosensor. The calibration curve showed that the peak current values (I_p) presented good linearity with the concentrations of S2 in the range from 5.0 f. to 5.0 pM with a regression equation of $I_p/\mu A=-0.3803\lg(C_{S2}/M)-0.5594$, $r=0.9937$. Based on 3σ (where σ is the standard deviation of the blank solution, $n=7$), the limit of detection for the target DNA was estimated to be 0.11 fM. The detection limit value were compared with the results obtained on the other biosensors that also using polymer films as DNA immobilization platform. The results are displayed in Table 1. From the comparison, it could be seen that the developed DNA biosensor exhibited the lowest detection limit for the target DNA.

Regeneration and reproducibility of the biosensor

The regeneration and reproducibility are important properties of a DNA biosensor. The reproducibility of the biosensor was monitored by eleven parallel electrochemical measurements

Table 1 The comparison of the analytical parameters with other DNA biosensors based on organic polymers

Polymer materials	Linear range (M)	LOD (M)	Refs.
Polypyrrole	8.0×10^{-11}~1.6×10^{-7}	1.6×10^{-11}	[26]
PTAE	1.0×10^{-7}~5.0×10^{-6}	1.0×10^{-9}	[27]
Polypyrrole nanofibers	5.0×10^{-8}~1.0×10^{-6}	2.0×10^{-8}	[28]
Polyaniline nanowires	2.1×10^{-12}~2.1×10^{-6}	3.3×10^{-13}	[29]
PDC	1.0×10^{-10}~1.0×10^{-5}	2.4×10^{-11}	[30]
pEBT	5.0×10^{-15}~5.0×10^{-12}	1.1×10^{-16}	This work

PTAE poly(thiophen-3-yl-acetic acid 1,3-dioxo-1,3-dihydro-isoindol-2-yl ester); *PDC* poly-2,6-pyridinedicarboxylic acid

for 3.0×10^{-12} M target DNA, and the results showed that a relative standard deviation (RSD) of 4.7 % ($n=11$) was estimated, exhibiting a high reproducibility of the constructed DNA biosensor. Regeneration experiment was performed by incubating in TE buffer solution at 90 °C for 10 min and then cooled with ice-water. The results showed that the biosensor could be successively used for at least ten times without loosing its hybridization and detection ability. This excellent performance was likely related to the co-contributions from the steady electropolymerization film of EBT on GCE and the tight sulfamide bond between the probe DNA and EBT on the electrode surface.

Conclusions

We describe here that a new electrochemical DNA biosensor was fabricated based on a sulfamide reaction. A layer of sulfonic group-contained film was first obtained on a glassy carbon electrode through electropolymerization in EBT solution and then covalently coupled with the amino group modified probe DNA. The immobilization method showed the features of facile, fast and low-cost. When the biosensor was applied for the detection of the target DNA using MB as a hybridization indicator, it showed good selectivity for discriminating totally complementary target sequence from single-base mismatched, three-base mismatched and non-complementary sequences. The quantitative analysis experiment showed that the complementary sequences could be detected in a linear range from 5.0 fM to 5.0 pM with a detection limit of 0.11 fM. This work brings a promising application of sulfonic dyes in the immobilization of the amino contained biolmolecules such as DNA, antigens/antibodies, proteins, and so on.

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