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A simple strategy of probe DNA immobilization by diazotization-coupling on self-assembled 4-aminothiophenol for DNA electrochemical biosensor

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ABSTRACT

A novel and simple strategy for fabricating of DNA electrochemical biosensor was developed based on covalent coupling of probe NH_2–ssDNA (S_1) on Au electrode that had been functionalized by diazotization of assembled 4-aminothiophenol (4-ATP) monolayer. The thiol group of 4-ATP allowed the stable assembly of 4-ATP monolayer. The following diazotization reaction was directly performed to prepare functional diazo–ATP film for covalent coupling of probe S_1. Remarkably, it was noting that the diazo–ATP provided a surface with high conductibility for electron transfer. The complementary ssDNA was determined by using differential pulse voltammetry. The linear range of the developed biosensor was from 1.57×10^{-9} to 4.52×10^{-7} M with a detection limit of 3.26×10^{-10} M. The fabricated biosensor possessed good selectivity and could be regenerated. The covalent immobilization of probe S_1 by simple diazotization-coupling on self-assembled 4-ATP monolayer could serve as a versatile platform for DNA immobilization and biosensors fabricating.

1. Introduction

With the development of biotechnology and medicine, simple and sensitive detection of target ssDNA provides potential in forensic, clinical, and pharmaceutical applications (Wang, 2006; Berganza et al., 2007). Various techniques have been proposed for DNA detection, e.g., fluorescent detection, surface plasmon resonance spectroscopy, electrochemistry and quartz crystal microbalance. Electrochemical detection, mainly based on DNA electrochemical biosensors, attracts considerable interest owning to advantages of simplicity, low-cost and high sensitivity (Li et al., 2008a; Piro et al., 2007; Lucarelli et al., 2008). Generally, the main target for the fabrication of DNA electrochemical biosensors is to prepare well defined DNA recognition interface by effective immobilization of probe ssDNA onto transducer surface through suitable matrix and immobilization method.

As a promising method for interfacial modification, self-assembling technique has gained increasing attention within fundamental and applied perspectives because it is allowed to control the surface coating at molecular scale. Due to the attractive features and inherent versatility, such technique can provide efficient way for creating advanced materials particularly with respect to the development of biosensors (Sánchez-Pomales et al., 2007; Guidotti et al., 2007; Jiao et al., 2005; Xu et al., 2007). The formation of self-assembled monolayers (SAMs) with well-organization and stability on a gold electrode by using compounds containing thiol group is versatile and efficient (Guidotti et al., 2007; Jiao et al., 2005; Bharathi et al., 2001). 4-Aminothiophenol (4-ATP) has been widely used as block for the construction of functionally self-assembled film. For example, it has been used to prepare analog of azobenzene. Some modified films show semiconducting properties and a phenomenon of phase transition (Jiao et al., 2005; Shaaban et al., 1993).

The objective of this work was to develop novel DNA electrochemical biosensor for sensitive detection of ssDNA. A new and simple strategy for fabricating of DNA electrochemical biosensor was described based on covalent immobilization of probe NH_2–ssDNA (S_1) by diazotization-coupling on self-assembled 4-ATP monolayer. As thiol groups of 4-ATP allowed sulfur–Au interaction, 4-ATP could stably assemble on the surface of Au electrode. Through diazotization interaction, diazo–ATP for covalent immobilization of probe S_1 was formed. In addition, diazo–ATP proved a surface with remarkable conductibility for electron transfer. Based on ssDNA immobilization by such diazotization-coupling, the fabricated biosensor showed promising performances e.g. high sensitivity, good selectivity and regeneration ability. The preparation methodology, analytical characteristic features were described and discussed in detail.

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2. Experimental

2.1. Reagents

4-ATP (Sigma–Aldrich) was used in this study. Tris(1,10-phenanthroline)cobalt(III) perchlorate, [$Co(phen)_3(ClO_4)_3$] was prepared as reported (Dollimore and Gillard, 1973). The 21-mer synthetic oligonucleotides for the HBV were purchased from SBS Genetech Company (Beijing, China). Their base sequences are as follows:

- immobilized probe NH_2–ssDNA (S_1): 5′-NH_2-AAT-GTG-CTC-CCC-CAA-CTC-CTC-3′;
- target ssDNA (S_2, complementary to S_1): 5′-GAG-GAG-TTG-GGG-GAG-CAC-ATT-3′;
- four-base mismatched ssDNA (S_3): 5′-GCG-GAG-CTG-AGG-GAG-CAT-ATT-3′.

S_2 and S_3 were dissolved in Tris–EDTA buffer (TE, pH 8.0) and S_1 were dissolved in PBS buffer (pH 7.0). The obtained solutions were kept frozen. All other chemicals were of analytical reagent grade and double distilled water (DDW) was used throughout.

2.2. Apparatus and instrumentations

Cyclic voltammetric (CV) and differential pulse voltammetric (DPV) measurements were performed with a CHI 832B electrochemical analyzer (Shanghai CH Instrument Company, China). Electrochemical impedance spectroscopy (EIS) was performed on a CHI 660C electrochemical analyzer (Shanghai CH Instrument Company, China). A three-electrode system was employed with Pt wire as auxiliary electrode, Ag/AgCl (saturated KCl) as reference electrode, and gold (Au) electrode or modified Au electrode as the working electrode.

2.3. Immobilization of ssDNA on self-assembled 4-ATP monolayer modified electrode by diazotization-coupling

Prior to modification, the working Au electrode was polished to a mirror-like surface with 1.0, 0.3, 0.05 μm alumina slurry on microcloth pads, and sonicated in ethanol and water. The freshly polished electrodes were then pretreated by cleaning with piranha solution (7:3 mixture of concentrated sulfuric acid and 30% hydrogen peroxide). Afterwards, the Au electrode was electrochemically cleaned by several potential cycling between −0.2 V and 0.6 V versus Ag/AgCl in 0.1 M H_2SO_4.

In present work, the preparing procedure of 4-ATP assembly and diazotization on a clean Au electrode (Jiao et al., 2005) was copied to the as-prepared Au electrode. In brief, the assembly of 4-ATP monolayer was performed by direct immersion of the electrode in ethanol solution containing 0.5 mM 4-ATP for 10 h. Then, the obtained electrode was completely cleaned with ethanol and water to remove unassembled thiol component. Afterwards, the 4-ATP-modified Au electrode was treated using the optimum procedure for diazotization (Jiao et al., 2005). Typically, the 4-ATP-modified Au electrode was transferred into 0.1 M HCl solution at 2–4 °C, and 100 mg $NaNO_2$ was slowly added with the total concentration of about 0.05 M, which is a little excess to ensure the completeness of the reaction. The reaction of diazotization was subsequently performed at the electrode surface. After 30 min incubation, the Au electrode was removed and immediately rinsed with icy water (Jiao et al., 2005).

For the covalent coupling of probe S_1, the obtained diazo–ATP/Au electrode was immersed into 20 mM HAc–NaAc buffer (pH 5.5) containing 26.43 μM NH_2–ssDNA (S_1) for 30 min at 2–4 °C. Thus, the obtained electrode was rinsed with the same HAc–NaAc buffer to eliminate non-specifically adsorbed S_1. After the electrode was air-dried, the probe S_1 modified electrodes was thus obtained and denoted as S_1/diazo–ATP/Au in the text.

2.4. DNA hybridization on probe S_1-modified electrode

The S_1/diazo–ATP-modified Au electrode was immersed in 20 mM Tris–HCl buffer (pH 7.0) containing different concentration of target S_2 or mismatched S_3 with shaking for 1 h at 37 °C. After hybridization, the obtained electrodes were washed with the same Tris–HCl buffer and water to remove non-specifically bound DNA. The electrodes thus obtained were denoted as S_1–S_2/diazo–ATP/Au and S_1–S_3/diazo–ATP/Au, respectively.

2.5. Indicator binding

$Co(phen)_3^{3+}$ possessed specific affinity for double stranded DNA (dsDNA). The hybridization between probe S_1 and target S_2 resulted in the formation of dsDNA on the electrode. $Co(phen)_3^{3+}$ was accumulated onto the dsDNA modified surface by immersing the electrode into the stirred 0.1 mM $Co(phen)_3^{3+}$ solution (0.1 M PBS, pH 6.5) for 10 min at 37 °C. The obtained electrodes were then rinsed with the same PBS for 10 s and denoted as Co–S_1–S_2/diazo–ATP/Au.

2.6. Electrochemical detection

The modified electrodes obtained during the self-assembled process were electrochemically characterized by using EIS, CV and DPV. EIS measurement was performed with the frequencies ranging from 10^4 to 1 Hz in 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture containing 0.1 M KCl. CV and DPV measurements were performed using 0.1 M PBS (pH 6.5) solution as the supporting solution.

2.7. Regeneration of the modified electrode

The electrodes obtained after DNA hybridization was regenerated by rinsing the hybridized surfaces with hot (95 °C) DDW for 5 min, followed by rapid cooling in icy bath. The reusability of the biosensor was tested by repetitive hybridization with target S_2 and regeneration (Li et al., 2008b).

3. Results and discussion

3.1. Immobilization of probe S_1 on self-assembled 4-ATP monolayer modified electrode by simple diazotization-coupling

Self-assembling technique has been proven as a promising method for the construction of interface with functionality and controllability (Zhang et al., 2008; Kumar and Chen, 2007; Song et al., 2008; Shervedani and Bagherzadeh, 2008; Loyprasert et al., 2008). DNA immobilization based on functionally self-assembled monolayer could be constructed as model system for the development of biosensors. In present investigation, probe S_1 was covalently coupled on Au electrode that had been functionalized by direct diazotization of self-assembled 4-ATP monolayer. Schematic representation of the fabrication of DNA biosensor based on covalent immobilization of probe S_1 on self-assembled diazo–ATP-modified Au electrode by diazotization-coupling was given in Fig. 1. As seen, three steps were involved in the covalent immobilization of ssDNA (Fig. 1A–C). Firstly, thiol group in 4-ATP allowed the assembly on Au electrode through sulfur–Au interaction (Jiao et al., 2005; Caldwell et al., 1995; Zhang et al., 2003). Secondly, the assembled 4-ATP monolayer was azo-derivatized. Thirdly, the probe S_1 was covalently coupled on diazo–ATP-modified Au electrode.

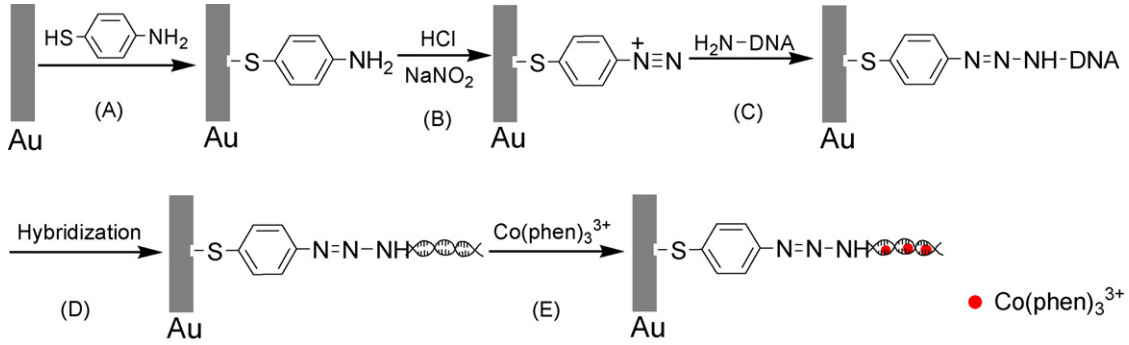

Fig. 1. Schematic representation of the fabrication of DNA biosensor based on covalent immobilization of probe S_1 on self-assembled diazo–ATP-modified Au electrode by diazotization-coupling.

3.2. Electrochemical characteristics of the developed DNA biosensor

In the developed DNA biosensor, probe S_1 was covalently immobilized on self-assembled ATP monolayer modified electrode through diazotization-coupling. The as-prepared biosensor was evaluated by CV. CVs of bare gold electrode (a), ATP/Au (b), Co–S_1/diazo–ATP/Au (c) and Co–S_1–S_2/diazo–ATP/Au (d) in a 0.1 M PBS (pH 6.5) at a scan rate of 50 mV s^{-1} were given in Fig. 2. It was obvious that the peak currents obtained on ATP/Au electrode were significantly lower (Fig. 2b) than those on the bare one (Fig. 2a). The phenomenon could be ascribed to the modification of Au electrode by forming a ATP monolayer. Compared with Co–S_1/diazo–ATP/Au (c) and Co–S_1–S_2/diazo–ATP/Au (d), a significant peak corresponded to $[Co(phen)_3]^{3+}$ appeared on Co–S_1–S_2/diazo–ATP/Au electrode (Fig. 2d). The results showed that the electrochemical indicator $[Co(phen)_3]^{3+}$ binds to dsDNA more efficiently than to ssDNA. The phenomenon further proved the formation of dsDNA on S_1–S_2/diazo–ATP/Au electrode and the incorporation of $[Co(phen)_3]^{3+}$ on the electrode surface.

CV of S_1–S_2/diazo–ATP/Au electrode in 0.1 M phosphate buffer solution (pH 6.5) containing 0.1 mM $[Co(phen)_3]^{3+}$ at different scan rates was also investigated. It was revealed that the peak-to-peak potential difference was almost independent of the scan rate over the entire range of scan rates. Such characteristic was similar with the result reported by Jiao et al. (2005). It should be mainly ascribed to the fact that the redox moiety, diazo, was coupled with a conjugated underlayer, 4-ATP layer, where can undergo an excellent electron transfer (Jiao et al., 2005).

EIS analysis was performed to provide further evidence for surface assembly on electrode and to study the interface properties of the developed biosensor. Fig. 3 showed the Nyquist plots obtained on gold electrode (a), ATP/Au (b), diazo–ATP/Au (c), S_1/diazo–ATP/Au (d), S_1–S_2/diazo–ATP/Au (e) and Co–S_1–S_2/diazo–ATP/Au (f) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$. As seen, significant differences in the electron transfer resistances (*R*et) were observed upon the stepwise modification of the electrode. For the bare gold electrode (Fig. 3a), the value of *R*et was found to be 516.4 Ω. After 4-ATP assembly on the surface of Au electrode, the value of *R*et increased to 1455 Ω, implying remarkable increase of the resistance to electron transfer of the electrochemical probe (Fig. 3b). When diazotization was performed, it was surprised to find that the diazo–ATP/Au electrode possessed a remarkably low *R*et of 0.01 Ω (Fig. 3c). The electron transfer between the electrochemical probe and electrode surface was highly strengthened. Such significant decrease might be ascribed to the excellent conductivity of the diazo–ATP (Jiao et al., 2005). It has been reported that diazoaminobenzene based on diazo–ATP showed semiconducting properties and phase transition and can chelate transition metals into metal complexes which show electrical and magnetic performances (Jiao et al., 2005). After probe S_1 was covalently coupled to diazo–ATP, the value of *R*et slightly increased to 0.05 Ω (Fig. 3d). After S_1–S_2 hybridization, the S_1–S_2/diazo–ATP/Au electrode exhibited a *R*et of 0.11 Ω (Fig. 3e). When the electrochemical indicator $[Co(phen)_3]^{3+}$ intercalated into the dsDNA, the *R*et of Co–S_1–S_2/diazo–ATP/Au significantly decreased and a 0.01 Ω was evaluated (Fig. 3f). On the basis of the EIS results, the assembly of

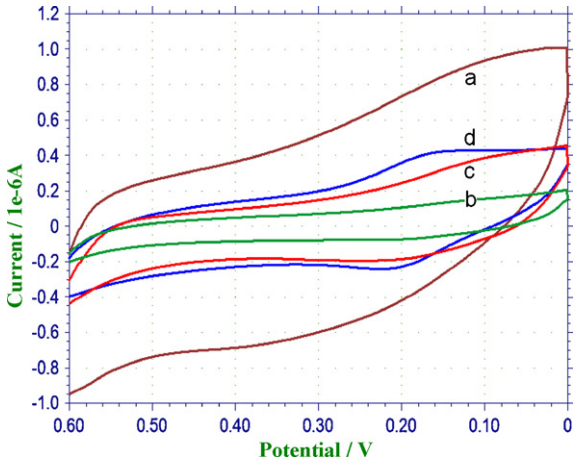

Fig. 2. CVs of bare gold electrode (a), ATP/Au (b), Co–S_1/diazo–ATP/Au (c) and Co–S_1–S_2/diazo–ATP/Au (d) in a 0.1 M PBS (pH 6.5) at a scan rate of 50 mV s^{-1}.

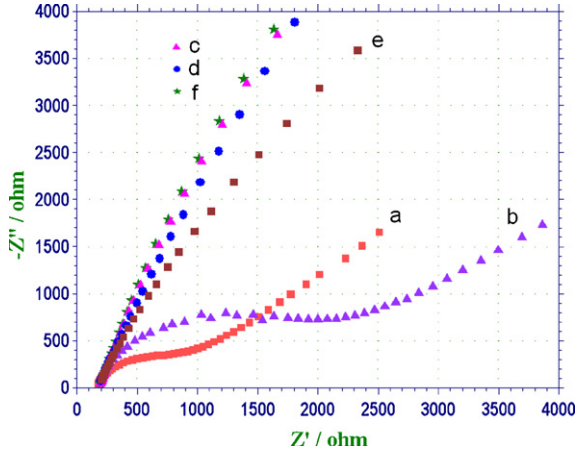

Fig. 3. EIS for bare gold electrode (a), ATP/Au (b), diazo–ATP/Au (c), S_1/diazo–ATP/Au (d), S_1–S_2/diazo–ATP/Au (e) and Co–S_1–S_2/diazo–ATP/Au (f) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$.

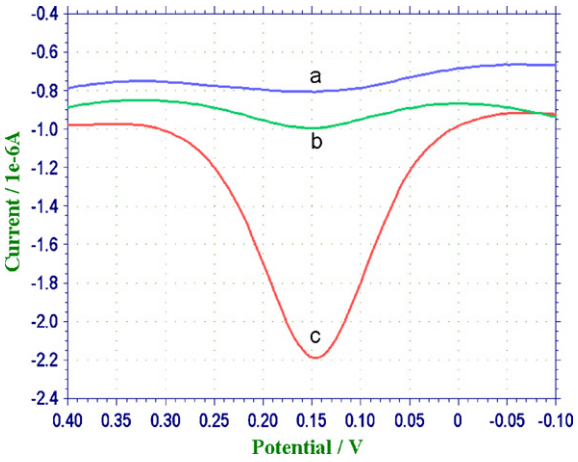

Fig. 4. DPVs of $[Co(phen)_3]^{3+}$ on S_1/diazo–ATP/Au (a), S_1–S_3/diazo–ATP/Au (b), S_1–S_2/diazo–ATP/Au (c) in 0.1 M PBS (pH 6.5). The concentration of S_2 and S_3 was 2.511×10^{-7} and 2.533×10^{-7} M, respectively.

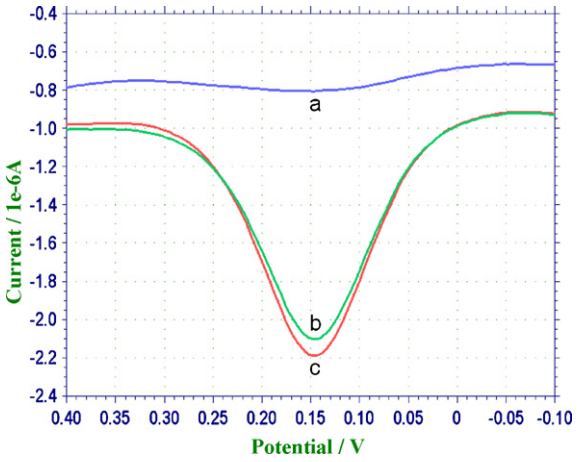

Fig. 5. DPVs of $[Co(phen)_3]^{3+}$ on a probe DNA regeneration/hybridization cycle in 0.1 M PBS (pH 6.5) including the original S_1/diazo–ATP/Au (a), Co–S_1–S_2/diazo–ATP/Au obtained after 12th regeneration/hybridization (b) and the original Co–S_1–S_2/diazo–ATP/Au (c).

4-ATP and the following diazo-derivatization were further proved. In addition, the successful immobilization of NH_2–ssDNA was concluded.

3.3. The selectivity of the developed biosensor and the determination of target S_2

The selectivity of the developed DNA electrochemical biosensor was investigated by using the S_1/diazo–ATP/Au electrode to hybridize with different kinds of DNA sequences including complementary ssDNA sequence (S_2) and non-complementary ssDNA sequence (S_3). Fig. 4 showed the DPV signals of $[Co(phen)_3]^{3+}$ on S_1/diazo–ATP/Au electrode (Fig. 4a) and after hybridization with complementary S_2 (Fig. 4c) and non-complementary DNA sequence S_3 (Fig. 4b). As seen, high signal was obtained with the S_1–S_2/diazo–ATP/Au electrode. Only very slight change in signal was observed after S_1/diazo–ATP/Au electrode hybridized with non-complementary S_3 sequence. The phenomenon showed high selectivity of the developed biosensor.

The sensitivity of the developed biosensor was investigated by varying the concentration of target S_2. The current obtained in DPV response of $[Co(phen)_3]^{3+}$ after S_1–S_2 hybridization was recorded with three repetitive measurements. The average peak current has a good linear relation versus concentration of S_2 in the range of 1.57×10^{-9} to 4.52×10^{-7} M, with a correlation coefficient of 0.9981. The regression equation is $\Delta i_p = 4.532C + 1.016 \times 10^{-8}$ (C, M; Δi_p, A). A detection limit of 3.26×10^{-10} M of the target S_2 can be estimated using 3σ (where σ is the standard deviation of the blank solution, $n = 11$). Compared with our previous work on the detection of HBV DNA, the linear range was wider and the detection limit was lower (Zhang et al., 2006, 2008; Li et al., 2007b, 2008b,c). It might be ascribed to the covalent immobilization of the probe ssDNA by diazotization-coupling on self-assembled ATP and the excellent conductivity of the Co–S_1–S_2/diazo–ATP/Au.

3.4. Regeneration of the developed biosensor

The S_1–S_2/diazo–ATP/Au electrodes were regenerated to investigate their reusability. Regeneration was accomplished by rinsing the hybridized surfaces with hot DDW for 5 min, followed by rapid cooling in icy bath. The reusability of the biosensor was tested by repetitive hybridization with complementary ssDNA and regeneration. DPVs of $[Co(phen)_3]^{3+}$ on DNA probe regeneration/hybridization cycle were given in Fig. 5. After 12 regeneration and hybridization, the electrode possessed 91.9% of its original current response (Fig. 5b and c). Therefore, the regeneration of the S_1/diazo–ATP/Au possessed potential for continuous monitoring of target DNA in the future.

4. Conclusion

The successful preparation of the DNA electrochemical biosensor demonstrated the efficiency for covalent immobilization of probe ssDNA via direct diazotization-coupling on assembled 4-ATP monolayer. The fabrication process offered simple and convenient methodology for functionality of self-assembly monolayer and covalent immobilization of NH_2–ssDNA. The developed DNA electrochemical biosensor possessed high sensitivity and regeneration ability. The promising performance of the developed DNA electrochemical biosensor makes this methodological study and application attractive in DNA analysis.

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