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Gold nanoparticles modified electrode via simple electrografting of in situ generated mercaptophenyl diazonium cations for development of DNA electrochemical biosensor

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ABSTRACT

A novel protocol for development of DNA electrochemical biosensor based on gold nanoparticles (AuNPs) modified glassy carbon electrode (GCE) was proposed, which was carried out by the self-assembly of AuNPs on the mercaptophenyl film (MPF) via simple electrografting of in situ generated mercaptophenyl diazonium cations. The resulting MPF was covalently immobilized on GCE surface via C–C bond with high stability, which was desirable in fabrication of excellent performance biosensors. Probe DNA was self-assembled on AuNPs through the well-known Au–thiol binding. The recognition of fabricated DNA electrochemical biosensor toward complementary single-stranded DNA was determined by differential pulse voltammetry with the use of $Co(phen)_3^{3+}$ as the electrochemical indicator. Taking advantage of amplification effects of AuNPs and stability of MPF, the developed biosensor could detect target DNA with the detection limit of 7.2×10^{-11} M, which also exhibits good selectivity, stability and regeneration ability for DNA detection.

1. Introduction

As one of the methods for covalent grafting of organic thin films for surface modification, the electrochemical grafting of aryldiazonium salts has received substantial attention in recent years for its simple procedure but versatile derivates (Pinson and Podvorica, 2005; Lyskawa and Bélanger, 2006; Malmos et al., 2009; Viel et al., 2008; Brooksby and Downard, 2005a,b). Generally speaking, covalent electrografting of aryldiazonium salts on underlying substrates has great advantage over traditional strategies with simple procedure, high stability, versatile functionality, and good controllability.

Aryldiazonium salts can be easily obtained through a classical diazotization reaction of primary amine of an aryl compound, which can be realized in nitrous acidic aqueous media or organic media. Then the aryldiazonium salts can be electrografted on carbon surfaces to form covalent C–C bond between the surface and organic layer via a one electron reduction process (Pinson and Podvorica, 2005). Although thiols can be self-assembled on gold electrode, leading to well-organized monolayers, the Au–S bond has a relatively low enthalpy (in the order of 170 kJ mol^{-1}) (Brooksby and Downard, 2005a). Notably, the specific functional groups on the aryl aromatic ring can impart desirable properties to the modified electrodes for practical applications (Pinson and Podvorica, 2005; Downard, 2000), and a significant number of aryl compounds with the functional groups of $-OCH_3$, $-NO_2$, –SH, –COOH, $-NH_2$, –CN, etc. were investigated (Viel et al., 2008; Harper et al., 2009; Allongue et al., 1997). Nowadays, the underlying substrates have been extended from carbon to various materials, such as molecule, macromolecule, polymer, carbon nanotubes, silicon, graphite, graphene, metallic particles and so on (Viel et al., 2008; Masheter et al., 2007; Allongue et al., 1997; Adenier et al., 2005; Laforgue et al., 2005; Pinson and Podvorica, 2005), which contributed to the development of material chemistry. Also, the formation of monolayer or multilayer and surface coverage can be controlled by varying the electrografting condition to meet special needs in particular cases (Laforgue et al., 2005; Pinson and Podvorica, 2005). Thus, the electrochemical grafting of aryldiazonium salts to form organic layer can be a good choice in electrochemical biosensor fabrication.

Gold nanoparticles (AuNPs) are one of the most promising nanomaterials which have high surface-to-volume ratio, unique catalytic activity, high electron transfer rate, and strong adsorption ability (Ding et al., 2004). They are good candidates for loading of biomolecules and improving the stability and immobilization

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amount of the attached biomolecules (Shulga et al., 2007; Xu et al., 2007; Manesh et al., 2008). For HS-DNA can be bound to the surface of gold substrates through the Au–thiol binding, AuNPs have been extensively used in the fabrication of DNA electrochemical biosensors (Hansen et al., 2006; Stoeva et al., 2006).

To the best of our knowledge, there has not been a report concerning the application of electrografting of in situ generated aryldiazonium salts in the fabrication of DNA electrochemical biosensor. In this paper, a novel strategy for AuNPs assembly on glassy carbon electrode (GCE) surface via a simple electrografting process was proposed and its application in DNA electrochemical biosensor was further investigated. 4-Aminothiophenol (4-ATP) was firstly diazotized with nitrous acid to form mercaptophenyl diazonium cations, and then the mercaptophenyl film (MPF) was electrografted on GCE surface. Afterwards, AuNPs were self-assembled on MPF through the Au-thiol binding. The effect of the concentration of mercaptophenyl diazonium cations on the electrografting was also investigated. Due to the high stability of the resulting MPF and amplification effects of AuNPs, the as-established DNA electrochemical biosensor displayed the excellent performances with high sensitivity, good selectivity and regeneration ability. The preparation methodology and analytical characteristic features were described and discussed in detail.

2. Experimental

2.1. Chemicals and materials

4-Aminothiophenol (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 by Dollimore and Gillard (1973). $HAuCl_4{\cdot}4H_2O$ was purchased from Sigma. The 21-mer synthetic oligonucleotides for the HBV were purchased from SBS Genetech. Company (Beijing, China). Their base sequences are as follows:

- Immobilized probe HS–ssDNA (S_1): 5′-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 (bold) ssDNA (S_3): 5′-G**C**G GAG **C**TG **A**GG GAG CA**T** ATT-3′

All oligonucleotides were dissolved in Tris–EDTA buffer solution (TE, pH 8.0) and kept frozen. Phosphate buffer solution (PBS) was used as the supporting electrolyte. All inorganic chemicals were of analytical grade.

2.2. Apparatus

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were performed with a CHI 832B electrochemical analyzer (Shanghai CH Instrument Company, China). Electrochemical impedance spectroscopy (EIS) measurements were carried out on a CHI 660C electrochemical workstation (Shanghai CH Instrument Company, China). A three-electrode system was employed with Pt wire as the auxiliary electrode, Ag/AgCl (saturated KCl) as the reference electrode, and GCE or modified GCE as the working electrode, respectively. X-ray photoelectron spectroscopy (XPS) measurement was performed on an ESCALAB–MKII spectrometer (VG Co., U.K.) with an Al K Alpha as the X-ray source for excitation. The data were obtained at room temperature, and typically the operating pressure in the analysis chamber was below 10^{-9} Torr with analyzer pass energy of 100 eV. The energy step size was 2.0 eV.

2.3. Preparation of AuNPs

All glassware used in the procedures was cleaned in a bath of freshly prepared solution of HNO_3–HCl (3:1, v/v), thoroughly washed with double distilled water (DDW), and dried prior to use. The 13-nm gold colloidal was prepared by the classical citrate reduction route for reduction of $HAuCl_4$ in aqueous solution (Xu et al., 2005; Frens, 1973).

2.4. Immobilization of probe DNA to MPF modified GCE

GCEs (3 mm in diameter) were polished with 1.0, 0.3, and 0.05 μm alumina slurry, respectively, rinsed thoroughly with DDW between each polishing step, then sonicated in 1:1 nitric acid solution (HNO_3/H_2O, v/v), acetone, and DDW successively and allowed to dry at room temperature.

Mercaptophenyl diazonium cations were generated based upon a procedure developed by Marshall and Mottola (Marshall and Mottola, 1983; Marshall and Mottola, 1985; Oklejas et al., 2008) with a little modification. 10 μmol 4-ATP was predissolved in methanol and then transferred into 5 mL of 0.5 M HCl solution. 10 μmol sodium nitrite ($NaNO_2$) was dissolved in 5 mL of DDW. Then the two reagents were mixed to form mercaptophenyl diazonium cations for 10 min at room temperature in the electrochemical cell. The electrografting was immediately performed in the diazotation mixture on GCE by one CV scan from 0.4 to −0.4 V at 100 mV s^{-1}. After the electrografting, the electrode was subsequently ultrasonicated in acetonitrile for 10 min to remove any adsorbed and loosely attached species on the surface. Then the electrode was subsequently thoroughly washed with ethanol and DDW and dried under N_2 stream. The modified electrode was thus obtained and denoted as MP/GCE. The resulting electrode, with a SH-riched surface, was immersed into AuNPs solution for 6 h at room temperature to obtain AuNPs modified electrode (AuNPs–MP/GCE).

For probe S_1 immobilization on AuNPs modified electrode, the above electrode was immersed into 20 mM HAc–NaAc buffer (pH 5.5) containing 26.35 μM HS–ssDNA for 6 h. Afterwards, the electrode was rinsed with the same HAc–NaAc buffer to eliminate non-specifically adsorbed S_1 and air-dried, the probe S_1 modified electrode was thus obtained and denoted as S_1/AuNPs–MP/GCE in the text.

2.5. DNA hybridization on probe S_1 immobilized electrode

The hybridization was conducted as follows: The S_1/AuNPs–MP/GCE 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 DDW to remove non-specifically bound DNA. The electrodes thus obtained were denoted as S_1–S_2/AuNPs–MP/GCE and S_1–S_3/AuNPs–MP/GCE, respectively.

2.6. Binding of indicator

$Co(phen)_3^{3+}$ was used as the indicator in the work for its specific affinity for double stranded DNA (dsDNA). The binding was realized by immersing the S_1–S_2/AuNPs–MP/GCE into the stirred 0.1 mM $Co(phen)_3^{3+}$ solution (0.1 M PBS, pH 7.0) for 10 min at 37 °C. The obtained electrode was then rinsed with the same PBS for 10 s and denoted as Co–S_1–S_2/AuNPs–MP/GCE.

2.7. Electrochemical detection

The modified electrodes obtained during stepwise modification were electrochemically characterized by using EIS, CV and DPV. EIS

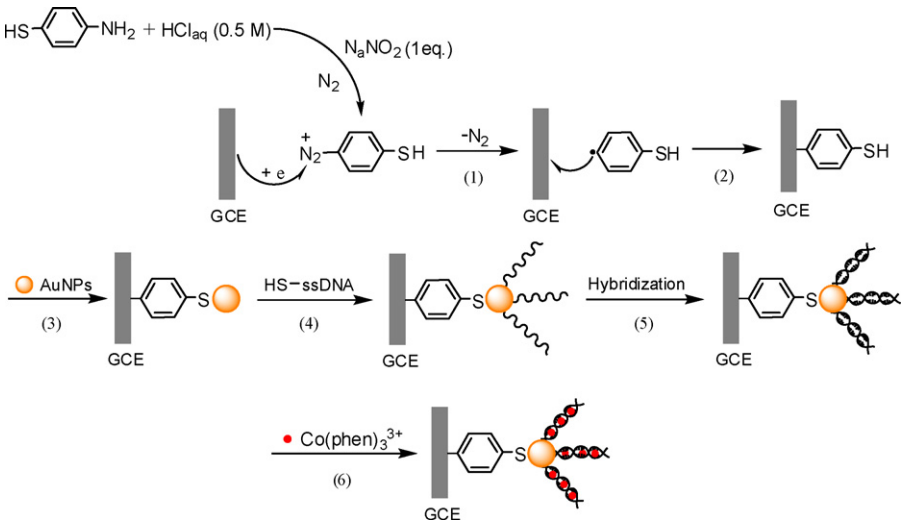

Fig. 1. Schematic representation of the fabrication of DNA biosensor.

measurement was performed with the frequency ranging from 10^4 to 1 Hz in 0.1 M KCl containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1). CV and DPV measurements were performed using 0.1 M PBS (pH 7.0) as the supporting solution.

2.8. Regeneration of the modified electrode

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

3. Results and discussion

3.1. Immobilization of probe ssDNA on AuNPs–MP/GCE

The electrografting of in situ generated mercaptophenyl diazonium cations on GCE surface affords the corresponding mercaptophenyl radicals, which can further react with carbon atoms of the carbon substrate to form covalent C–C bond. The mechanism of electrografting mercaptophenyl diazonium cations to carbon surface can be described as follows:

$$C + SH\text{–}Ar\text{–}N_2^+ + e^- \rightarrow C\text{–}Ar\text{–}SH + N_2$$

Based on the principle, a DNA electrochemical biosensor was fabricated and the detailed modification process is illustrated in Fig. 1. Firstly, 4-ATP molecules were diazotized with nitrous acid to form mercaptophenyl diazonium cations, and then MPF was grafted on GCE surface by a simple electrochemical reduction process. With the generation of mercaptophenyl diazonium cations, the solution turned to orange which is distinguished from colorless of the original state, indicating the successful diazotization of 4-ATP molecules. Subsequently, AuNPs were chemisorbed onto the SH-riched surface by the Au–thiol binding. Finally, probe S_1 was immobilized on the surface of AuNPs–MP/GCE for DNA hybridization detection.

MPF was grafted on GCE surface via CV in 2 mL diazotation mixture of 1.0 mM $NaNO_2$ and 1.0 mM 4-ATP in 0.5 M HCl at a scan rate of 100 mV s^{-1} and the obtained CVs are shown in Fig. 2. It is obvious that a broad irreversible wave located at a potential close to 0 V appeared at the first scan, corresponding to the formation of mercaptophenyl radicals by reduction of the in situ generated of mercaptophenyl diazonium cations. The wave reached to a point of almost complete disappearance on the subsequent scans, indicating the electrode surface had been modified and passivated by the formed MPF (Malmos et al., 2009; Adenier et al., 2005). For comparison, there is no reduction peak observed in the curve obtained by scanning the bare GCE in mixture of 1.0 mM $NaNO_2$ and 0.5 M HCl without 4-ATP (Fig. 2b), indicating the successful electrografting of mercaptophenyl diazonium cations on GCE surface.

AuNPs can be easily self-assembled on the mercaptophenyl moiety via the Au–thiol binding, which was demonstrated by CV obtained at AuNPs–MP/GCE in 0.1 M H_2SO_4. Fig. 3 shows the voltammetric response of MP/GCE (a) and AuNPs–MP/GCE (b) over the potential range of 0–1.5 V at a scan rate of 100 mV s^{-1}. Compared with MP/GCE (Fig. 3a), a typical CV of a well-defined surface of gold electrode was obtained on AuNPs–MP/GCE (Fig. 3b), with the peak potential located at 0.85 V and 1.38 V, respectively. It is obvious that AuNPs were successfully attached to the GCE surface.

The effect of mercaptophenyl diazonium cations concentration on electrografting has also been investigated (see Fig. S1 in the supplementary material). Fig. S1 shows CVs of MP/GCE prepared in different concentration of diazotation mixtures in 0.1 M KCl containing 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1). The concentration was 0, 0.5, 1.0, 4, and 8 mM, respectively, and the interaction time was 10 min. For no MPF modification on GCE, the $Fe(CN)_6^{3-/4-}$

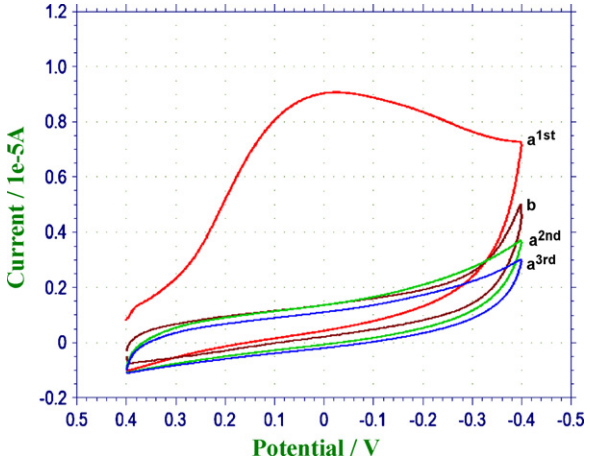

Fig. 2. CVs of GCE in diazotation mixture (1.0 mM $NaNO_2$ 1.0 mM 4-ATP in 0.5 M HCl) (a^{1st}) first cycle, (a^{2nd}) second cycle, and (a^{3rd}) third cycle. (b) CV of GCE in 1.0 mM $NaNO_2$ + 0.5 M HCl free of diazonium salt at a scan rate 100 mV s^{-1}.

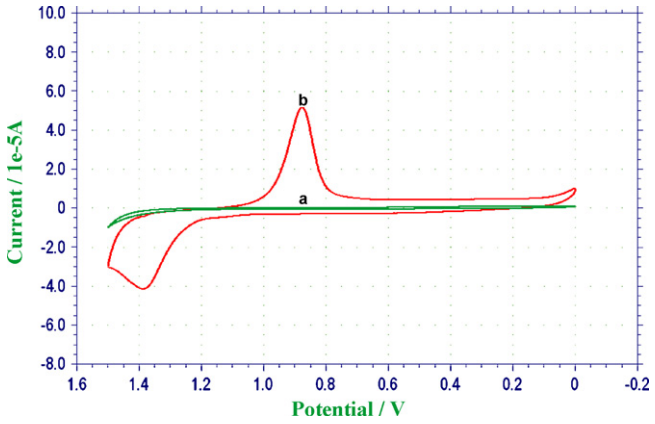

Fig. 3. CVs of the MP/GCE (a) and AuNPs–MP/GCE (b) in 0.1 M H_2SO_4 at a scan rate of 100 mV s^{-1}.

redox couple presents a reversible system located at 0.185 and 0.225 V, respectively. With the increase of the concentration of mercaptophenyl diazonium cations, the $Fe(CN)_6^{3-/4-}$ redox couple showed a slowness electrochemical response on modified electrodes. The anodic peak potential and the cathodic peak potential were shifted toward the positive and the negative direction of potential, respectively. Also the peak currents decreased gradually. This can be attributed to the repulsion of the hydrophobic organic layer and the hydrophilic probe, which prevents the redox couple from approaching the electrode surface. At low concentration, the layer was thin and inhomogeneous. As the concentration increased, the layer became compact. Taking the electron transfer rate into consideration, 1.0 mM diazotation mixture was selected in the experiment.

3.2. XPS analysis of differently modified GCEs

The attachment of mercaptophenyl radicals to GCE surface has been proven by XPS analysis as shown in Fig. 4. The characteristic peak of C 1s occurred at about 284.6 eV was observed for the bare glassy carbon (GC) plate (a), MP/GC plate (b), and AuNPs–MP/GC plate (c). Compared with the bare GC plate (Fig. 4a), the characteristic peak of S 2p located at about 164.2 eV which can be attributed to SH appeared at MP/GC plate (Fig. 4b), indicating the successful electrografting of mercaptophenyl radicals on GCE surface. Another peak appeared at 83.4 eV, companied with a shoulder peak of 87.9 eV on the AuNPs–MP/GC plate (Fig. 4c), which were undoubtedly corresponded to the peaks of Au 4f, indicating the

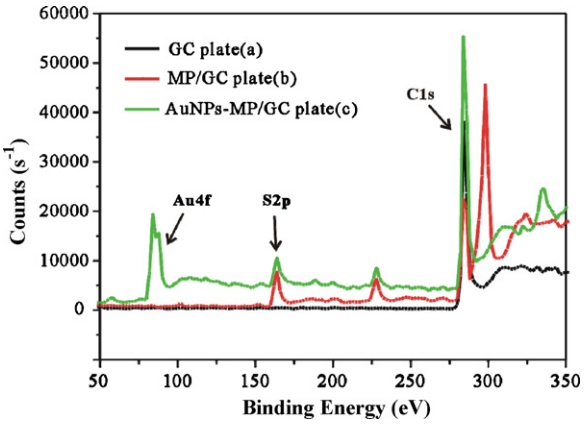

Fig. 4. XPS survey spectra of bare GC plate (a), MP/GC plate (b), and AuNPs–MP/GC plate (c).

presence of AuNPs on the MP/GC plate. The characteristic peak of S 2p on AuNPs–MP/GC plate was about 162.9 eV, which was attributed to the formation of Au–S bond. It further demonstrated that AuNPs have been assembled on the MP/GC plate.

3.3. Electrochemical characteristics of the developed DNA biosensor

In the developed DNA electrochemical biosensor, probe S_1 was self-assembled on AuNPs modified electrode, which is based on the simple electrografting of in situ generated mercaptophenyl diazonium cations on electrode surface. Voltammetric responses of bare GCE (a), MP/GCE (b), AuNPs–MP/GCE (c), S_1/AuNPs–MP/GCE (d), and Co–S_1–S_2/AuNPs–MP/GCE (e) in 0.1 M PBS (pH 7.0) at a scan rate of 50 mV s^{-1} are recorded (see Fig. S2 in the supplementary material). After MPF was electrografting on GCE surface, the current of the resulting MP/GCE is lower than that of bare GCE. This phenomenon is probably due to the block effect of MPF on the electron transfer, which was also demonstrated by the fact that the electrode surface has been passivated by the resulting MPF in Fig. 2. As soon as AuNPs were modified on electrode surface, the current increased dramatically, indicating that AuNPs could enhance the electron transfer remarkably. Compared with probe ssDNA immobilized on electrode surface, a significant pair of redox peaks appeared on Co–S_1–S_2/AuNPs–MP/GCE with the anodic and cathodic potential of 0.24 V and 0.09 V, respectively. This could be definitely attributed to the redox reaction of $[Co(phen)_3]^{3+}$, which demonstrated the formation of dsDNA on S_1–S_2/AuNP–MP/GCE and the incorporation of $[Co(phen)_3]^{3+}$ on the electrode surface. CVs of Co–S_1–S_2/AuNPs–MP/GCE in 0.1 M PBS (pH 7.0) at different scan rates were 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, which is consistent with the result of our previous work (Zhang et al., 2005).

EIS was employed to provide further information of different electrode surfaces during the modified process and to study the interfacial properties of the developed biosensor. Fig. 5 shows the Nyquist plots obtained on bare GCE (a), MP/GCE (b), AuNPs–MP/GCE (c), S_1/AuNPs–MP/GCE (d), S_1–S_2/AuNPs–MP/GCE (e), and Co-S_1–S_2/AuNPs–MP/GCE (f) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$. After MPF was electrografted on the surface of GCE, in curve b, the value of electron transfer resistance (R_{et}) increased to 2400 Ω, which is much

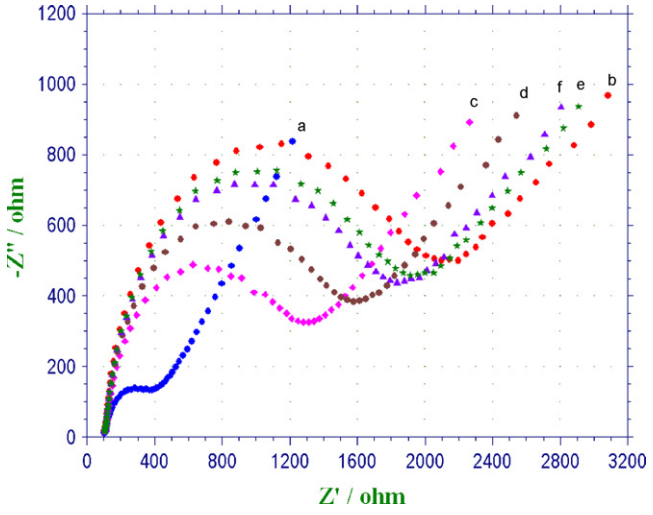

Fig. 5. EIS for bare GCE (a), MP/GCE (b), AuNPs–MP/GCE (c), S_1/AuNPs–MP/GCE (d), S_1–S_2/AuNPs–MP/GCE (e), and Co–S_1–S_2/AuNPs–MP/GCE (f) in 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$.

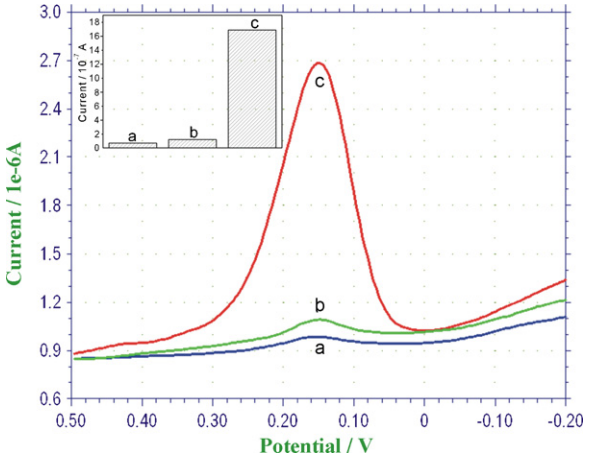

Fig. 6. DPVs of $[Co(phen)_3]^{3+}$ on S_1/AuNPs–MP/GCE (a), S_1–S_3/AuNPs–MP/GCE (b), S_1–S_2/AuNPs–MP/GCE (c) in 0.1 M PBS (pH 7.0). The concentration of S_2 and S_3 was 1.0×10^{-8} M and 1.0×10^{-8} M, respectively. Inset shows histograms of the above DPV signals.

larger than that of the bare GCE (Fig. 5a). This phenomenon can be attributed to the surface passivated by the formed MPF hindered the electron transfer between the GCE surface and the redox-probe. As soon as AuNPs were self-assembled on the electrode surface, the value of R_{et} decreased to 1400 Ω (Fig. 5c), implying that AuNPs may play a role similar to a conducting wire or electron-conducting tunnel to improve the electron transfer ability (Zhang et al., 2005). After S_1 was immobilized on AuNPs–MP/GCE, the R_{et} increased (Fig. 5d) due to the electrostatic repulsion between the negatively charged $Fe(CN)_6^{3-/4-}$ and DNA phosphate backbone. The repulsion effect was further enhanced (Fig. 5e) after hybridization was completed on the electrode surface, however, the R_{et} of Co–S_1–S_2/AuNPs–MP/GCE significantly decreased when the electrochemical indicator $[Co(phen)_3]^{3+}$ intercalated into the dsDNA (Fig. 5f). The EIS results indicated that modification and hybridization were successfully accomplished on the electrode surface.

3.4. The selectivity, regeneration of the developed biosensor and the determination of target S_2

The selectivity of the developed DNA electrochemical biosensor was investigated by using $Co(phen)_3^{3+}$ as an electrochemical hybridization label for detection of different DNA sequences. Seen from Fig. 6, a negligible DPV peak current of 0.0691 μA was obtained for $Co(phen)_3^{3+}$ at the potential of 0.24 V for the ssDNA immobilized electrode, indicating that $Co(phen)_3^{3+}$ has a very weak affinity for probe DNA by electrostatic interaction on the modified electrode surface (Fig. 6a). After S_1/AuNPs–MP/GCE hybridized with S_3, the signal change of $Co(phen)_3^{3+}$ at S_1–S_3/AuNPs–MP/GCE was calculated to be 0.0536 μA (Fig. 6b) due to the absence of entire hybridization between the probe and four-base mismatched DNA. However, a pronounced increase in the current value (16.83 μA) of $Co(phen)_3^{3+}$ was obtained when incubating in S_2 sequence (Fig. 6c). The electrochemical response of hybrid with four-base mismatched DNA sequence is only 0.32% of that of complete complementary DNA sequence, showing a negligible effect of nonspecific DNA on detection of target DNA. Consequently, the developed biosensor possessed good selectivity.

The peak current change of $Co(phen)_3^{3+}$ on the Co–S_1–S_2/AuNPs–MP/GCE increased with the increasing target DNA concentration and exhibited a linear correlation to the target DNA concentration ranged from 1.1×10^{-10} to 4.0×10^{-8} M, with a correlation coefficient of 0.9974. The regression equation is $\Delta i_p = 0.1319C + 0.3285$ (C, 10^{-9} M; Δi_p, 10^{-6} A). A detection limit of 7.2×10^{-11} M of the target S_2 can be estimated using 3σ (where σ is the standard deviation of the blank solution, $n = 11$) (see Fig. S3 in the supplementary material). Compared with our previous work on the detection of HBV DNA, the linear range was wider and the detection limit was lower (Li et al., 2008, 2009; Zhang et al., 2007, 2008). It might be ascribed to the high stability and compact structure of MPF on electrode surface, which favored more AuNPs self-assembly and subsequent probe DNA loading.

Regeneration ability is another important factor of monitoring the performance of the developed biosensor and the regeneration of the developed DNA electrochemical biosensor was investigated (see Fig. S4 in the supplementary material). The electrode possessed 95.3% of its original current response when the fabricated biosensor was regenerated 12 times by rinsing the hybridized surface with hot DDW for 5 min, followed by rapid cooling in icy bath. It shows that this proposed DNA electrochemical biosensor has good regeneration ability for DNA detection. Therefore, the regeneration of the S_1/AuNPs–MP/GCE possessed potential for continuous monitoring of target DNA.

4. Conclusions

By utilizing a simple electrograting of in situ generated mercaptophenyl diazonium cations on GCE surface, a novel and effective route for fabrication of DNA electrochemical biosensor based on AuNPs assembly on the resulting MPF was demonstrated. MPF was immobilized on surface via C–C bond, thus diminished the loss of probe ssDNA during the experiment process and contributed to the excellent performance of the developed biosensor. This procedure provides an easy, inexpensive methodology for functionalizing GCE and should be easily extended for more applications toward other biomolecules analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.07.076.

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