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Gold nanoparticles modified electrode via a mercapto-diazoaminobenzene monolayer and its development in DNA electrochemical biosensor

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ABSTRACT

A novel protocol for the gold nanoparticles (AuNPs) modification on the electrode surface was proposed, which was based on the self-assembly of AuNPs on the mercapto-diazoaminobenzene monolayer modified electrode. The mercapto-diazoaminobenzene monolayer was obtained by covalent immobilization of 4-aminothiophenol (4-ATP) molecules onto another 4-ATP monolayer functionalized gold electrode by diazotization-coupling reaction. The DNA immobilization and hybridization on the AuNPs modified electrode was further investigated. The prepared AuNPs–ATP–diazo-ATP film demonstrated efficient electron transfer ability for the electroactive species toward the electrode surface due to a large conjugated structure of the mercapto-diazoaminobenzene monolayer. The recognition of fabricated electrochemical DNA biosensor toward complementary single-stranded DNA was determined by differential pulse voltammetry with the use of $Co(phen)_3^{3+}$ as an electrochemical indicator. A linear detection range for the complementary target DNA was obtained from 3.01×10^{-10} to 1.32×10^{-8} M with a detection limit of 9.10×10^{-11} M. The fabricated biosensor also possessed good selectivity and could be regenerated easily.

1. Introduction

DNA electrochemical biosensor has attracted considerable attention in recent years, which provides a sensitive, accurate, rapid and cost-effective platform for a variety of practical applications in the fields of medical diagnostics, clinical genetic analysis, forensic identification and environmental monitoring (Debouck and Goodfellow, 1999; Hanrahan et al., 2004; Luo et al., 2008). Generally, the most concerned issue in the fabrication of DNA electrochemical biosensor is the amount and the stability of immobilized probe single-stranded DNA (ssDNA) as well as the accessibility of target DNA toward the probe DNA immobilized electrode (Liu et al., 2005; Cederquist et al., 2008; Kjällman et al., 2008). It has been well elaborated that the immobilization amount and the molecular orientation of probe ssDNA could remarkably influence the operational performance of DNA electrochemical biosensor (Liu et al., 2008; Basuray et al., 2009). Therefore, numerous different immobilization strategies have been proposed and employed aimed at improving the link stability between DNA and transducer surface (Cederquist et al., 2008), or increasing the amount of immobilized DNA (Liu et al., 2005), and sometimes simplifying the immobilization procedure (Kjällman et al., 2008).

Gold nanoparticles (AuNPs) are one class of the nanomaterials widely used in DNA or protein biosensor fabrication for the enhancement of biosensor performance (Liu et al., 2005; Fu et al., 2005; Patolsky et al., 2001, 2002; Nebling et al., 2004; Dharuman et al., 2005; Elsholz et al., 2009). The self-assembly of AuNPs on the electrode surface could be easily achieved via the use of a bi-functional chemical linking agent such as 1,6-hexanedithiol, cysteamine, sol–gel, etc. Although these self-assembly methods are very simple and rapid, the formed monolayer on the electrode surface are usually insulated or could not offer a good electrical conductivity between AuNPs and electrode surface, which is not especially favorable for the fabrication of electrochemical sensor or biosensor. In this context, the development of novel and simple strategy for the immobilization of AuNPs that could achieve an effective electrical conductivity or well electron transfer ability in electrochemical sensor or biosensor fabrication is highly desirable.

Diazonium organic salt has been extensively used for the surface derivatization for a wide range of metal and semiconductor materials (Radi et al., 2009; Jiao et al., 2005). Diazonium groups functionalized electrode is of special interest owing to its ability to further react with phenolic, imidazole, or amino groups to form covalent diazo bonds for the achievement of different types

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of surface derivatization (Hermanson, 2008; Radi et al., 2009). For example, Radi et al. (2009) reported a novel protocol for covalent immobilization of horseradish peroxidase on gold electrode surface by diazotization-coupling reaction.

In our research, a novel strategy for AuNPs assembly on the electrode surface was proposed and its application in DNA electrochemical biosensor was further investigated. The 4-aminothiophenol (4-ATP) was firstly self-assembled on the gold electrode via the Au–S bond. After amine group was diazotized with nitrous acid, the mercapto-diazoaminobenzene monolayer modified electrode could be obtained following the reaction of diazonium group with amine group of another ATP. The thiol group was simultaneously introduced and could be used for the assembly of AuNPs on the electrode surface. This fabrication strategy for the AuNPs assembly was convinced to be able to improve the electron transfer ability of electroactive species toward the electrode surface owing to a big conjugated structure of mercapto-diazoaminobenzene monolayer. The as-established DNA biosensor displayed the excellent performances with high sensitivity, good selectivity and regeneration ability. This study essentially offers a facile and effective route for the AuNPs modification and fabrication of DNA electrochemical biosensor.

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). $HAuCl_4{\bullet}4H_2O$ 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′-HS-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′

All oligonucleotides were dissolved in Tris–EDTA buffer solution (TE, pH 8.0) and kept frozen. All other chemicals were of analytical reagent grade.

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 electrode or modified gold electrode as the working electrode, respectively.

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 water, and dried prior to use. The 13-nm gold colloidal solution was prepared by the classical citrate reduction route for the reduction of $HAuCl_4$ in aqueous solution (Xu et al., 2005; Frens, 1973).

2.4. Immobilization of probe DNA to the mercapto-diazoaminobenzene monolayer modified Au electrode

The gold electrode was polished with 1.0, 0.3, and 0.05 μm alumina slurry and soaked in an ultrasonic bath successively with double distilled water (DDW), absolute alcohol, and DDW. Then, the gold electrode was dipped in piranha solution (H_2SO_4/H_2O_2, 7:3, v/v) and electrochemically treated by cycling the potential between 0 and +1.5 V in 0.5 M H_2SO_4 until a stable gold oxide CV was obtained.

The freshly pretreated electrode was immersed into the 4-ATP (2.0 mM) solution for 6 h, where the assembly of 4-ATP monolayer was performed via sulfur–Au reaction. Then, the obtained electrode was completely cleaned with ethanol and DDW to remove unassembled thiol component.

The diazotization of 4-ATP on a clean Au electrode was performed according to the following procedure (Li et al., 2009). In brief, 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 a 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 electrode was removed and immediately rinsed with ice water. The as-prepared electrode was denoted as diazo-ATP/Au.

For covalent coupling of other 4-ATP molecules, the above diazo-ATP/Au was immersed into methanol (pH 5.0) containing 2.0 mM 4-ATP for 40 min at 2–4 °C. Afterwards, the obtained electrode was rinsed with the same solution to eliminate non-specifically adsorbed 4-ATP molecules. Then the electrode was dried under N_2 stream and denoted as ATP–diazo-ATP/Au. The surface of ATP–diazo-ATP/Au electrode, now containing exposed thiol moieties, was dipped into 13-nm AuNPs solution for 6 h at room temperature to obtain self-assembled AuNPs modified electrode (AuNPs–ATP–diazo-ATP/Au). Subsequently, a droplet of 10 μl 26.35 μM HS–ssDNA solution was dropped onto the surface of AuNPs–ATP–diazo-ATP/Au to realize the immobilization of S_1. Then the electrode was treated by air-drying, followed by rinsing with 20 mM Tris–HCl buffer solution (pH 7.0) to eliminate non-specifically adsorbed S_1. The probe ssDNA-modified electrode was thus obtained and denoted as S_1/AuNPs–ATP–diazo-ATP/Au in the text.

2.5. DNA hybridization on probe S_1-immobilized electrode

The hybridization was conducted as follows: the S_1/AuNPs–ATP–diazo-ATP/Au was immersed into 20 mM Tris–HCl buffer solution (pH 7.0) containing different concentrations 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 solution and DDW to remove non-specifically bound DNA. The resulted electrodes were denoted as S_1–S_2/AuNPs–ATP–diazo-ATP/Au and S_1–S_3/AuNPs–ATP–diazo-ATP/Au, respectively.

2.6. Binding of indicator

$Co(phen)_3^{3+}$ possesses 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 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–ATP–diazo-ATP/Au.

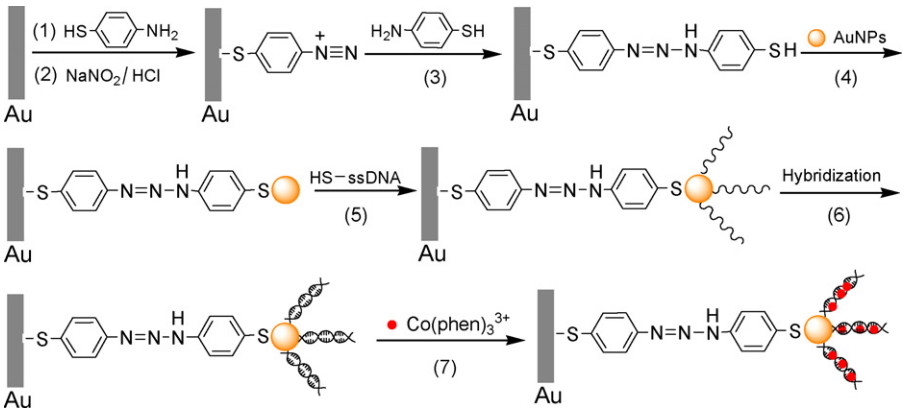

Fig. 1. Schematic representation for the fabrication of DNA biosensor.

2.7. Electrochemical detection

The modified electrodes obtained during stepwise modification were electrochemically characterized by using EIS, CV and DPV. EIS measurement was performed with the frequencies 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) solution as the supporting solution.

2.8. Regeneration of DNA biosensor

The electrodes obtained after DNA hybridization were regenerated by rinsing the hybridized surfaces with hot (95 °C) DDW for 5 min, followed by a rapid cooling in an ice 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 S_1 on AuNPs–ATP–diazo-ATP/Au

Molecular self-assembly has become a popular surface derivatization procedure owing to its simplicity, versatility, and high organization and homogeneity (Kumar and Chen, 2007; Shervedani and Bagherzadeh, 2008).

The detailed fabrication process of electrochemical DNA biosensor is illustrated in Fig. 1. Firstly, the amine group of self-assembled 4-ATP monolayer on Au electrode was conducted for diazo-derivatization (Li et al., 2009). Secondly, the mercapto-diazoaminobenzene monolayer was obtained via in situ diazotization-coupling reaction between diazo and amino group of another ATP molecule (Jiao et al., 2005). Then, AuNPs were chemisorbed onto the thiol group of the mercapto-diazoaminobenzene monolayer. Finally, probe S_1 was immobilized on the surface of AuNPs–ATP–diazo-ATP-modified Au electrode for DNA hybridization detection.

3.2. Electrochemical characteristics of the developed DNA biosensor

In the developed DNA biosensor, probe S_1 was immobilized on self-assembled AuNPs which were attached to the mercapto-diazoaminobenzene monolayer. The performance of the working Au electrode during stepwise modification was evaluated by CV in 0.1 M PBS (pH 7.0). CVs of ATP/Au (a), ATP–diazo-ATP/Au (b), AuNPs–ATP–diazo-ATP/Au (c) and Co–S_1–S_2/AuNPs–ATP–diazo-ATP/Au (d) in a 0.1 M PBS (pH 7.0) at a scan rate of 50 mV s^{-1} are given in Fig. 2. After the assembled 4-ATP monolayer was azo-derivatized and reacted with amino groups of other 4-ATP molecules to form diazo bonds, in curve b, the current is larger than that of ATP/Au (Fig. 2a), indicating that the ATP–diazo-ATP film processes a surface with high electrical conductibility for electron transfer. Compared with ATP–diazo-ATP/Au, the peak current in curve c increased dramatically, which confirmed that AuNPs could enhance the electron transfer remarkably. It was obvious that a significant pair of peaks corresponding to $[Co(phen)_3]^{3+}$ appeared on Co–S_1–S_2/diazo-ATP/Au electrode (Fig. 2d), which proved the formation of dsDNA on S_1–S_2/diazo-ATP/Au electrode and the successful incorporation of $[Co(phen)_3]^{3+}$ on the electrode surface. CV of S_1–S_2/AuNPs–ATP–diazo-ATP/Au electrode in 0.1 M PBS (pH 7.0) 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 Li et al. (2009). It should be mainly ascribed to the fact that the redox moiety, diazoaminobenzene, which possesses semiconducting properties, can undergo an excellent electron transfer (Shaaban et al., 1993; Jiao et al., 2005).

EIS was employed to provide further evidence for interface properties on electrode surface during stepwise modification. Fig. 3 shows the Nyquist plots obtained on bare gold electrode (a), ATP/Au (b), diazo-ATP/Au (c), ATP–diazo-ATP/Au (d), AuNPs–ATP–diazo-ATP/Au (e), S_1–S_2/AuNPs–ATP–diazo-ATP/Au

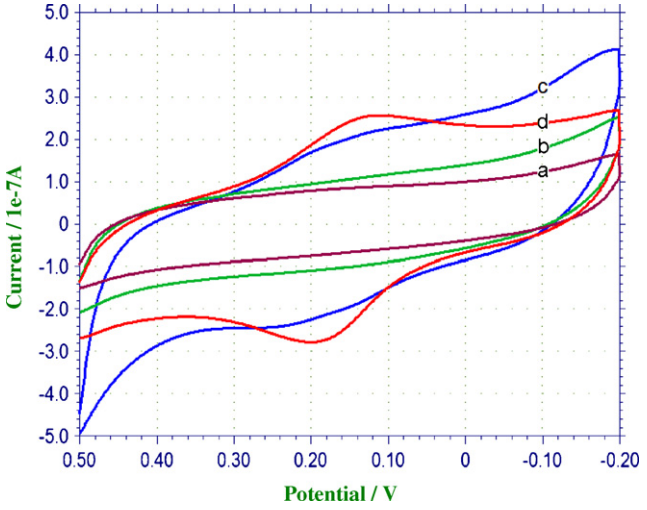

Fig. 2. CVs of ATP/Au (a), ATP–diazo-ATP/Au (b), AuNPs–ATP–diazo-ATP/Au (c) and Co–S_1–S_2/AuNPs–ATP–diazo-ATP/Au (d) in a 0.1 M PBS (pH 7.0) at a scan rate of 50 mV s^{-1}.

(f), and Co–S_1–S_2/AuNPs–ATP–diazo-ATP/Au (g) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$. As shown in Fig. 3, there were significant differences in the electron transfer resistance (R_{et}) upon the stepwise modification of the modified electrode. In comparison, the R_{et} of ATP/Au (Fig. 3b) was found to be 3500 Ω, which is much larger than that of bare Au electrode (Fig. 3a), implying the assembled 4-ATP film could obstruct the electron transfer of the electrochemical probe toward electrode surface. When diazotization was performed, the diazo-ATP/Au electrode possessed a remarkably low R_{et} (Fig. 3c). These results coincided well with our previous research, which reflected that the diazo-ATP film had excellent conductivity for easier electron transfer between the electrode surface and the redox probe (Li et al., 2009). Afterwards, the resulting diazo-ATP film covalently coupled to other 4-ATP molecules to form mercapto-diazoaminobenzene monolayer, the value of R_{et} slightly increased (Fig. 3d). This phenomenon could be attributed to semiconducting properties of the diazoaminobenzene units as a result of high molecular resonance induced by intermolecular hydrogen bonding at the diazo linkage (Shaaban et al., 1993). After AuNPs were attached to the electrode surface, it could be seen that the R_{et} value decreased dramatically (Fig. 3e), implying that AuNPs may play an important role as a conducting wire or electron-conducting tunnel in the electron transfer process (Zhang et al., 2005). After S_1–S_2 hybridization, the R_{et} of S_1–S_2/AuNPs–ATP–diazo-ATP/Au electrode increased (Fig. 3f), due to the electrostatic repulsion between the negatively charged $Fe(CN)_6^{3-/4-}$ and DNA phosphate backbone. When the electrochemical indicator $[Co(phen)_3]^{3+}$ intercalated into the dsDNA, as we expected, the R_{et} of Co–S_1–S_2/AuNPs–ATP–diazo-ATP/Au significantly decreased, indicating that the double helix with indicator may be served as a good electronic delivery channel in the biosensor (Fig. 3g). EIS results indicated that modification and hybridization were successfully accomplished on the electrode surface.

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/AuNPs–ATP–diazo-ATP/Au electrode to hybridize with different kinds of DNA sequences including target S_2 and non-complementary S_3. Fig. 4 shows the DPV signals of $[Co(phen)_3]^{3+}$ on S_1/AuNPs–ATP–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/AuNPs–ATP–diazo-ATP/Au electrode and there was very slight change in signal after S_1/AuNPs–ATP–diazo-ATP/Au electrode hybridized with non-complementary S_3 sequence. The phenomenon showed high selectivity of the developed biosensor.

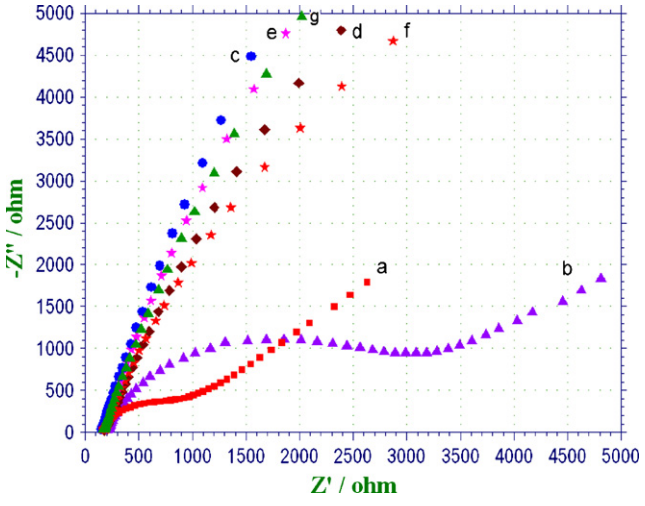

Fig. 3. EIS for bare gold electrode (a), ATP/Au (b), diazo-ATP/Au (c), ATP–diazo-ATP/Au (d), AuNPs–ATP–diazo-ATP/Au (e), S_1–S_2/AuNPs–ATP–diazo-ATP/Au (f) and Co–S_1–S_2/AuNPs–ATP–diazo-ATP/Au (g) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$.

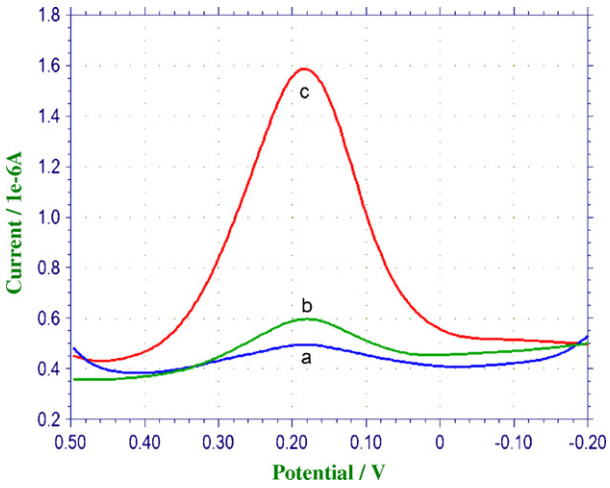

Fig. 4. DPVs of $[Co(phen)_3]^{3+}$ on S_1/AuNPs–ATP–diazo-ATP/Au (a), S_1–S_3/AuNPs–ATP–diazo-ATP/Au (b) and S_1–S_2/AuNPs–ATP–diazo-ATP/Au (c) in 0.1 M PBS (pH 7.0). The concentration of S_2 and S_3 was 2.222×10^{-9} and 2.233×10^{-9} M, respectively.

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 3.01×10^{-10} to 1.32×10^{-8} M, with a correlation coefficient of 0.9932. The regression equation is $\Delta i_p = 4.927C + 1.732 \times 10^{-8}$ (C, 10^{-2} M; Δi_p, A). A detection limit of 9.10×10^{-11} M of the target S_2 can be estimated using 3σ (where σ is the standard deviation of the blank solution, $n = 11$). 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., 2007, 2008; Li et al., 2008, 2009). It might be attributed to the immobilization of the probe S_1 onto the mercapto-diazoaminobenzene monolayer via AuNPs, both of which possessed excellent conductivity on the Co–S_1–S_2/AuNPs–ATP–diazo-ATP/Au.

The reproducibility was tested by repetitive hybridization with the use of 2.222×10^{-9} M S_2. The proposed biosensor showed an acceptable repeatability with a relative standard deviation (R.S.D.) of 3.9% for 11 successive assays. The long-term stability of the biosensor was investigated over 1 month storage and the biosensor retained more than 97.4% of its initial current response, demonstrating the stabilization effect of the mercapto-diazoaminobenzene monolayer for the immobilization of probe DNA.

3.4. Regeneration of the developed biosensor

The S_1–S_2/AuNPs–ATP–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 are given in Fig. 5. After 12 regeneration and hybridization, the electrode possessed 96% of its

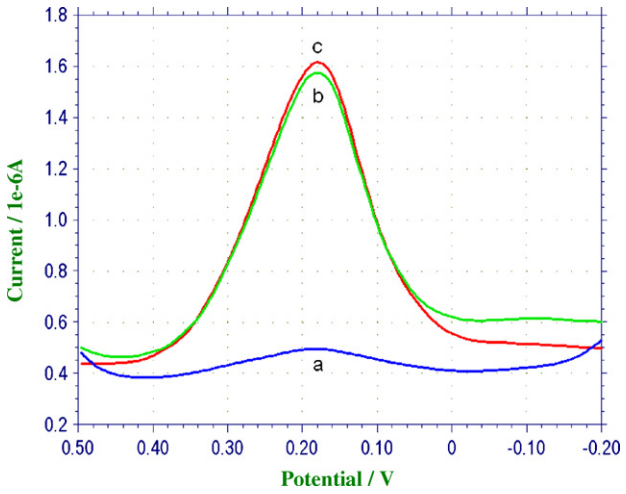

Fig. 5. DPVs of $[Co(phen)_3]^{3+}$ on a DNA probe regeneration/hybridization cycle in 0.1 M PBS (pH 7.0). The original ssDNA probe (a), 12th regeneration of the DNA probe (b) and hybridization (c).

original current response (Fig. 5b and c). Therefore, the regeneration of the S_1/AuNPs–ATP–diazo-ATP/Au possessed potential for continuous monitoring of target DNA in the future.

4. Conclusions

By utilizing the excellent electrical conductivity ability of mercapto-diazoaminobenzene monolayer, a novel and effective route for AuNPs assembly on electrode surface was demonstrated here. The fabricated DNA electrochemical biosensor on the AuNPs modified electrode exhibited a high detection sensitivity, good selectivity and regeneration ability. The current fabrication protocol should be easily extended for more applications toward other biomolecules analysis.

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