Biosensors and Bioelectronics 24 (2008) 781–786

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Development of DNA electrochemical biosensor based on covalent immobilization of probe DNA by direct coupling of sol–gel and self-assembly technologies

Feng Li, Wei Chen, Shusheng Zhang*

Key Laboratory of Eco-Chemical Engineering, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China

ARTICLE INFO

Article history:
Received 14 March 2008
Received in revised form 28 June 2008
Accepted 30 June 2008
Available online 10 July 2008

Keywords:
Biosensor
DNA
Covalent immobilization
Sol–gel
Self-assembly

ABSTRACT

A new procedure for fabricating deoxyribonucleic acid (DNA) electrochemical biosensor was developed based on covalent immobilization of target single-stranded DNA (ssDNA) on Au electrode that had been functionalized by direct coupling of sol–gel and self-assembled technologies. Two siloxanes, 3-mercaptopropyltrimethoxysiloxane (MPTMS) and 3-glycidoxypropyltrimethoxysiloxane (GPTMS) were used as precursors to prepare functionally self-assembly sol–gel film on Au electrode. The thiol group of MPTMS allowed assembly of MPTMS sol–gel on gold electrode surface. Through co-condensation between silanols, GPTMS sol–gel with epoxide groups interconnected into MPTMS sol–gel and enabled covalent immobilization of target NH_2–ssDNA through epoxide/amine coupling reaction. The concentration of MPTMS and GPTMS influenced the performance of the resulting biosensor due to competitive sol–gel process. The linear range of the developed biosensor for determination of complementary ssDNA was from 2.51×10^{-9} to 5.02×10^{-7} M with a detection limit of 8.57×10^{-10} M. The fabricated biosensor possessed good selectivity and could be regenerated. The covalent immobilization of target ssDNA on self-assembled sol–gel matrix could serve as a versatile platform for DNA immobilization and fabrication of biosensors.

1. Introduction

Rapid and simple determination of specific sequences of deoxyribonucleic acid (DNA) at low concentration is currently important in clinical, forensic and pharmaceutical applications (Mateo-Martí et al., 2007; Soper et al., 2006). Since electrochemistry possesses attractive features over other existing measurement systems, electrochemical biosensors promise to provide a simple, accurate and inexpensive platform for DNA analysis (Fu et al., 2005; Pänke et al., 2007). One of the key steps for fabrication of DNA electrochemical biosensors is effective combination of single-stranded DNA (ssDNA) layers with electrochemical transducers. To improve the performance of biosensors, effective immobilization of probe ssDNA onto the transducer surface through suitable method is of great significance.

During the past few decades, many efforts have been devoted to explore new supporting materials and immobilization methods for the fabrication of DNA biosensors. An emerging area that has attracted increased attention in recent years is the development of biosensors based on sol–gel-derived platforms. Sol–gel matrices possessed chemical inertness, physical rigidity, high thermal stability and negligible swelling in aqueous and organic solvents (Wang and Zhang, 2006; Liu and Sun, 2007; Wang et al., 2004; Massari et al., 2006). However, the majority of sol–gel-based biosensors focused on the entrapment of biomolecules for biosensor fabricating (Gill and Ballesteros, 2000; Wang et al., 2005). In general, entrapment techniques are easy to perform, but the bonding of the biomolecules is often weak. As a result, biomolecules can leach out from the electrode and be lost into the bulk solution. The phenomenon may lead to significant signal loss and affect the stability of the biosensor. Recently, there has been growing interest in covalent attachment of biomolecules to sol–gel matrices by using organic siloxane precursors with functional groups such as amino, glycidoxy and epoxy groups. Due to the attractive features and inherent versatility, such sol–gel method can provide versatile way for creating advanced materials particularly with respect to the development of biosensors (Liu et al., 2004, 2005; Shirosaki et al., 2005; Li et al., 2006; F. Li et al., 2007; Yang et al., 2003; Thenmozhi and Narayanan, 2007a,b; Zhang et al., 2007). With the development of self-assembly technology, functionally self-assembly sol–gel layers on electrodes could be constructed as model systems for the construction of DNA biosensors by covalent immobilization of target ssDNA.

* Corresponding author. Fax: +86 532 84022750.
E-mail address: shushzhang@qust.edu.cn (S. Zhang).

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doi:10.1016/j.bios.2008.06.052

In this investigation, a procedure for fabricating DNA electrochemical biosensor was described based on covalent immobilization of target ssDNA by direct coupling of sol–gel and self-assembled technologies. 3-Mercaptopropyltrimethoxysiloxane (MPTMS) and 3-glycidoxypropyltrimethoxysiloxane (GPTMS) were used as functional precursors to prepare functional sol–gel film. As thiol groups of MPTMS allowed sulfur–Au interaction, MPTMS sol–gel could assemble on the surface of Au electrode. Through co-condensation between silanols, GPTMS sol–gel interconnected into MPTMS sol–gel and provided epoxide groups for covalent immobilization of target NH_2–ssDNA through epoxide/amine coupling reaction. Due to competitive condensation in sol–gel process, the concentration of MPTMS and GPTMS significantly influenced the performance of the resulting biosensor. The fabricated biosensor showed promising performance, e.g., high sensitivity, good selectivity and regeneration ability. The preparation methodology, main characteristic features were described and discussed in detail.

2. Experimental

2.1. Reagents

MPTMS (Alfa aesar) and GPTMS (Alfa aesar) were 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). Three 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 mismatch ssDNA (S_3): 5′-G<u>C</u>G-GAG-<u>C</u>TG-<u>A</u>GG-GAG-CA<u>T</u>-ATT-3′.

All oligonucleotides were dissolved in Tris–EDTA buffer (TE, pH 8.0) and 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). 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. Electrochemical impedance spectroscopy (EIS) was performed on a CHI 660C electrochemical analyzer (Shanghai CH Instrument Company, China). Saturated calomel electrode (SCE) was used as the reference electrode in EIS investigation.

2.3. Immobilization of ssDNA on self-assembled MPTMS–GPTMS sol–gel-modified Au electrodes

The Au electrode was polished to a mirror-like surface with 1.0, 0.3 and 0.05 μm alumina slurry on microcloth pads, and sonicated in ethanol and water. The freshly polished electrodes were pretreated by cleaning with piranha solution (7:3 mixture of concentrated sulfuric acid and 30% hydrogen peroxide). After electrochemical cleaning by several potential cycling between −0.2 V and 0.6 V versus Ag/AgCl in 0.1 M H_2SO_4, Au electrode was immersed in a mixture of MPTMS/GPTMS for a certain time to produce a self-assembled sol–gel film (MPTMS–GPTMS). Afterwards, the probe ssDNA-modified electrode was obtained by transferring 20 μL of 26.43 μM NH_2–ssDNA (S_1) solution onto the surface of MPTMS–GPTMS/Au. Then the electrode was followed by air-drying until the surface was dry. The obtained electrode was then rinsed with 20 mM Tris–HCl buffer (pH 7.0) to eliminate the non-specifically adsorbed S_1. The target ssDNA-modified electrodes thus obtained were denoted as S_1/MPTMS–GPTMS/Au in the text.

2.4. Hybridization on target S_1-modified electrode

The S_1/MPTMS–GPTMS-modified Au electrode was immersed in 20 mM Tris–HCl buffer (pH 7.0) containing different concentrations of target (S_2) or mismatch (S_3) ssDNA 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/MPTMS–GPTMS/Au and S_1–S_3/MPTMS–GPTMS/Au, respectively.

2.5. Indicator binding

The $Co(phen)_3^{3+}$ 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 PBS for 10 s and denoted as Co–S_1–S_2/MPTMS–GPTMS/Au.

2.6. Electrochemical detection

The electrochemical characteristics of the modified electrode obtained during the self-assembled process were characterized by using EIS, CV and DPV. EIS measurement was performed in 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture containing 0.1 M KCl with the frequencies ranging from 10^4 to 10^{-1} Hz. CV and DPV measurements were performed in 0.1 M PBS (pH 6.5) solution or 0.1 M PBS (pH 6.5) containing 0.1 mM $Co(phen)_3^{3+}$ solution, respectively.

2.7. Regeneration of the modified electrode

The electrodes obtained after DNA hybridization were regenerated by rinsing the hybridized surfaces with hot (95 °C) DDW for 5 min, followed by rapid cooling in ice bath. The reusability of the biosensor was tested by repetitive hybridization with target ssDNA and regeneration (X.M. Li et al., 2007).

3. Results and discussion

3.1. Immobilization of target ssDNA on self-assembled MPTMS–GPTMS sol–gel electrodes

The technique based on molecular self-assembly attracts increased attention in recent years because it is allowed to control the architecture of surface coating (Zhang et al., 2008). Sol–gel technology provides unique means to prepare matrix with a three-dimensional network (Wang and Zhang, 2006; Liu and Sun, 2007; Wang et al., 2004; Massari et al., 2006). Functionally self-assembly sol–gel film could be constructed as model systems for DNA immobilization in the development of biosensors. In the present investigation, target ssDNA was covalently immobilized on Au electrode that had been functionalized by direct coupling of sol–gel and self-assembled technologies. Two functional siloxanes, MPTMS and GPTMS were used as precursors for sol–gel process. Schematic representation for the development of DNA biosensor based on

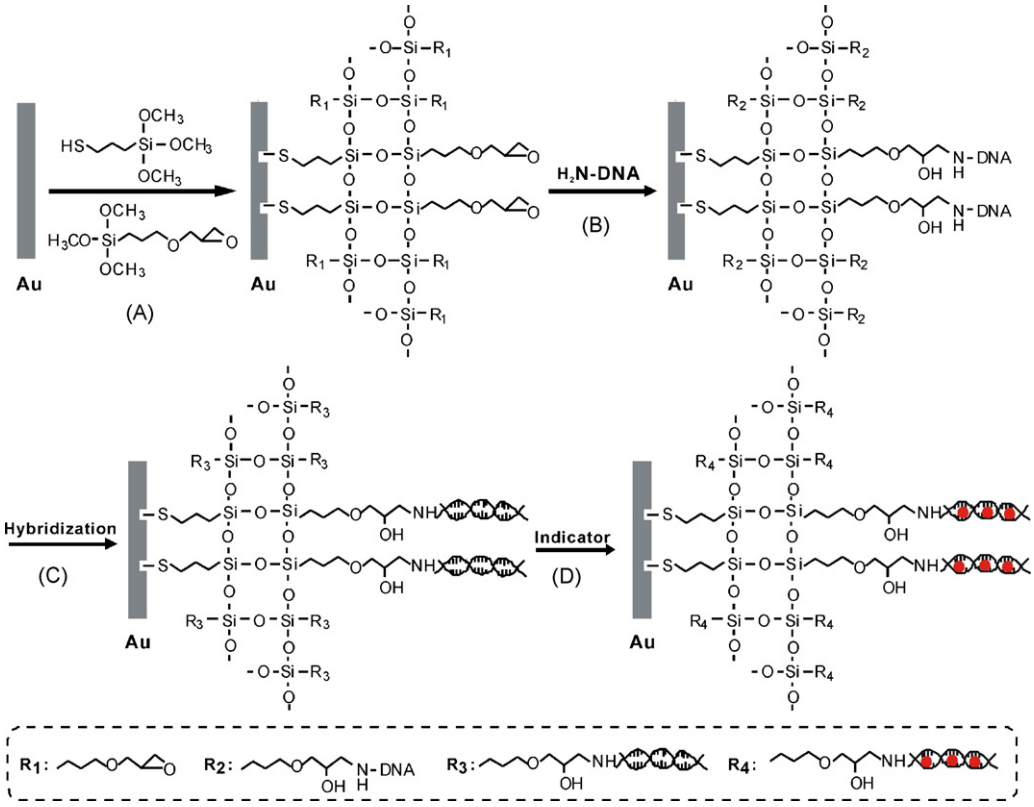

Fig. 1. Schematic representation for the development of DNA biosensor based on covalent immobilization of target NH_2–ssDNA on self-assembled MPTMS–GPTMS sol–gel-modified Au electrode.

covalent immobilization of target NH_2–ssDNA on self-assembled MPTMS–GPTMS sol–gel-modified Au electrode is given in Fig. 1. As seen, the covalent immobilization of ssDNA involved three aspects. Firstly, thiol group in MPTMS allowed the assembly on Au electrode through sulfur–Au interaction. As a result, MPTMS sol–gel layer with full of SH could stably self-assemble on Au electrode and possess highly ordered and well-organized structures, as reported by Fu et al. (2005) (del Pozo et al., 2005). Secondly, hydrolyzable methoxy groups in MPTMS allowed the covalent binding of functional GPTMS to MPTMS sol–gel through co-condensation between silanols. As known, silanols were formed through self-hydrolysis of MPTMS and GPTMS. Consequently, GPTMS sol–gel containing full of epoxy groups could interconnect with MPTMS sol–gel. Thirdly, epoxy groups in GPTMS sol–gel enabled covalent immobilization of target ssDNA through epoxide–amine coupling reaction between epoxide groups in GPTMS sol–gel and the amino linker of the ssDNA (NH_2–ssDNA) (Fu et al., 2005; Li et al., 2006; F. Li et al., 2007; Macanovic et al., 2004).

3.2. Effect of the concentration of MPTMS/GPTMS and gelation time

The characteristics of the self-assembled MPTMS–GPTMS sol–gel could be easily controlled by changing parameters of sol–gel reaction such as molar ratio of MPTMS/GPTMS and the gelation time. The influence of concentration of MPTMS/GPTMS on the electrochemical performance of the developed biosensor was investigated (see supplementary material). The gelation time was all controlled at 14 h. When the concentration of MPTMS was fixed at 5 mM or 1 mM, the concentration of GPTMS changed significantly. As seen, the biosensor prepared using 5 mM MPTMS showed low electrochemical response compared with those obtained using 1 mM MPTMS. Though the same 1 mM MPTMS was used, different GPTMS concentrations resulted in different electrochemical performances. In low concentration, current response increased as the concentration of GPTMS increased. When the concentration of GPTMS was 20 mM, the current arrived at a maximum value. Afterwards, the current response decreased when the concentration of GPTMS increased. We view that the concentration of MPTMS/GPTMS might result in a competitive sol–gel reaction due to the competitive condensation between silanols. When MPTMS/GPTMS was mixed, silanol groups were in situ generated through self-hydrolysis. Then, self-condensation and co-condensation between silanols from MPTMS and GPTMS, and addition of amino groups of NH_2–ssDNA to epoxy rings took place simultaneously. When the concentration of MPTMS increased, the content of GPTMS in the final MPTMS–GPTMS decreased. As NH_2–ssDNA was covalently linked to epoxide group, the higher the concentration of GPTMS, the higher amount of NH_2–ssDNA immobilization. As a result, a relatively low concentration of MPTMS and high concentration of GPTMS was needed. However, too high concentration of GPTMS might result in a much thick sol–gel film and most of NH_2–ssDNA was buried inside the thick sol–gel bulk. As a result, the electron transfer could not effectively occur and the sensitivity of the developed biosensor decreased (Guo et al., 1995). According to the electrochemical performance of the developed biosensor, the concentration of MPTMS/GPTMS of 1:20 (mM) was selected for further investigation.

The gelation time was optimized from 2 h to 36 h (see supplementary material). As shown, the current response of the developed biosensor increased from a 2 h gelation time and reached the maximum at a 14 h gelation time. Then, the current response of the developed biosensor decreased. Therefore, the suitable gelation time for the maximum electrochemical performance was at

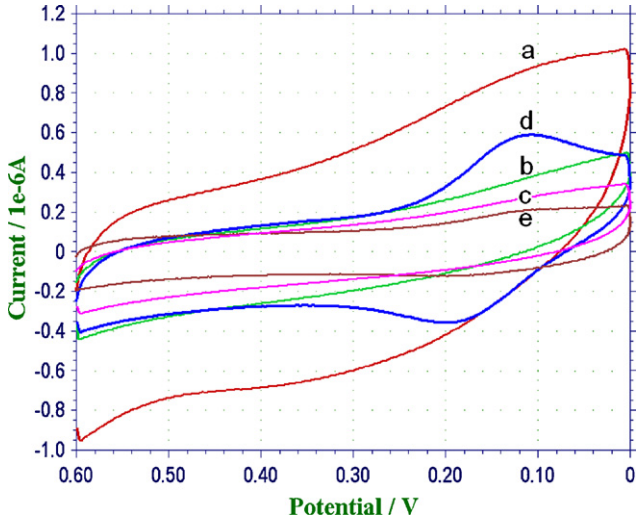

Fig. 2. CVs of bare gold electrode (a), MPTMS–GPTMS/Au (b), S_1/MPTMS–GPTMS/Au (c), Co–S_1–S_2/MPTMS–GPTMS/Au (d) and S_1–S_2/MPTMS–GPTMS/Au (e) in a 0.1 M PBS (pH 6.5) at a scan rate of 50 mV s^{-1}.

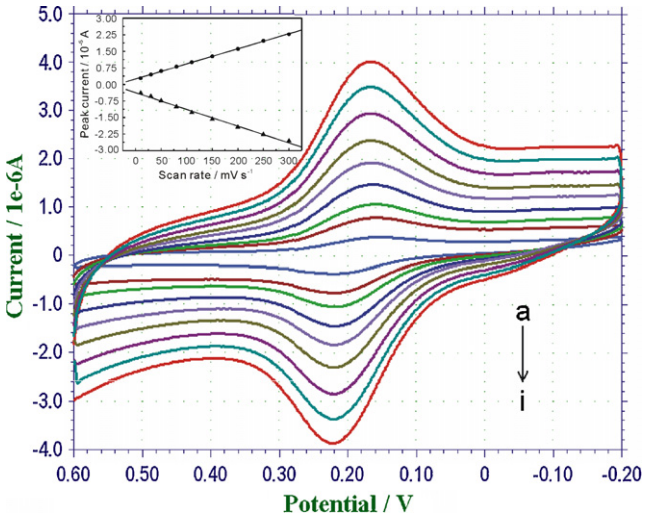

Fig. 3. CVs of S_1–S_2/MPTMS–GPTMS/Au in 0.1 M PBS (pH 6.5) containing 0.1 mM $[Co(phen)_3]^{3+}$ at different scan rates of 10 mV s^{-1} (a), 30 mV s^{-1} (b), 50 mV s^{-1} (c), 80 mV s^{-1} (d), 110 mV s^{-1} (e), 150 mV s^{-1} (f), 200 mV s^{-1} (g), 250 mV s^{-1} (h) and 300 mV s^{-1} (i). Inset shows the plots of peak current versus the scan rate.

14 h. In the preparation of self-assembled MPTMS–GPTMS sol–gel-modified electrode, sol–gel and self-assembled technologies were coupled. When the gelation time was low, the binding between MPTMS sol–gel and Au might be weak. On the contrary, too long gelation time resulted in thick sol–gel film and decreased the sensitivity of the developed biosensor (Guo et al., 1995).

3.3. Electrochemical characteristics of the developed DNA biosensor

The developed DNA biosensor with target NH_2–S_1 covalent immobilization on self-assembled MPTMS–GPTMS sol–gel was evaluated by cyclic voltammetry. Cyclic voltammograms (CVs) of bare gold electrode (a), MPTMS–GPTMS/Au (b), S_1/MPTMS–GPTMS/Au (c) and S_1–S_2/MPTMS–GPTMS/Au (d) in a 0.1 M PBS (pH 6.5) containing 0.1 mM $[Co(phen)_3]^{3+}$ were investigated (see supplementary material). The MPTMS–GPTMS/Au, S_1/MPTMS–GPTMS/Au and S_1–S_2/MPTMS–GPTMS/Au electrodes had the same E_{pa} of 0.217 V. However, the E_{pc} were 0.154 V, 0.156 V and 0.166 V, respectively. Therefore, E_{pc} shifted to positive potentials when $[Co(phen)_3]^{3+}$ interacted with S_1–S_2/MPTMS–GPTMS/Au electrode. As S_2 was complementary to the immobilized target S_1, dsDNA could be formed on the electrode. Incorporation of $[Co(phen)_3]^{3+}$ on the surface of S_1–S_2/MPTMS–GPTMS/Au electrode through intercalative binding was deduced (Carter et al., 1989; del Pozo et al., 2005). CVs of bare Au electrode (a), MPTMS–GPTMS/Au (b), S_1/MPTMS–GPTMS/Au (c), Co–S_1–S_2/MPTMS–GPTMS/Au (d) and S_1–S_2/MPTMS–GPTMS/Au (e) in a 0.1 M PBS (pH 6.5) are given in Fig. 2. These CVs can prove that MPTMS–GPTMS sol–gel had self-assembled onto the Au electrode. At the same time, it indicated that the sol–gel film blocked the electron transfer. As seen, a significant peak corresponding to $[Co(phen)_3]^{3+}$ appeared for Co–S_1–S_2/MPTMS–GPTMS/Au electrode. The phenomenon further proved the formation of dsDNA on S_1–S_2/MPTMS–GPTMS/Au electrode and the incorporation of $[Co(phen)_3]^{3+}$ on the electrode surface. The incorporation of $[Co(phen)_3]^{3+}$ on S_1–S_2 was also performed using UV spectrum. Hypsochromic shift and hyperchromic effect was observed after $[Co(phen)_3]^{3+}$ interacted with a solution containing both S_1 and S_2 (data not shown in brief).

Typical CVs of S_1–S_2/MPTMS–GPTMS/Au electrode in 0.1 M phosphate buffer solution (pH 6.5) containing 0.1 mM $[Co(phen)_3]^{3+}$ at different scan rates are shown in Fig. 3. It was clear that the peak potential was independent of the scan rate over the entire range of scan rates. From this result, we confirmed that S_1–S_2/MPTMS–GPTMS/Au had good electrochemical reversibility. Inset showed the plots of peak current versus the scan rate. As seen, the peak current was proportional to the scan rate, indicating that peak currents were surface-confined.

EIS analysis was performed to provide further evidence for interface assembly on electrode and to study the interface properties of the developed biosensor. Fig. 4 showed the Nyquist plots obtained on bare Au electrode (a), MPTMS–GPTMS/Au (b), S_1/MPTMS–GPTMS/Au (c), Co–S_1–S_2/MPTMS–GPTMS/Au (d) and S_1–S_2/MPTMS–GPTMS/Au (e). As seen, significant differences in the electron transfer resistances (R_{et}) were observed upon the stepwise formation of the modified electrode. The bare gold electrode almost exhibited a straight line (Fig. 4a), which was characteristic of a diffusion limited electrochemical process. After MPTMS–GPTMS

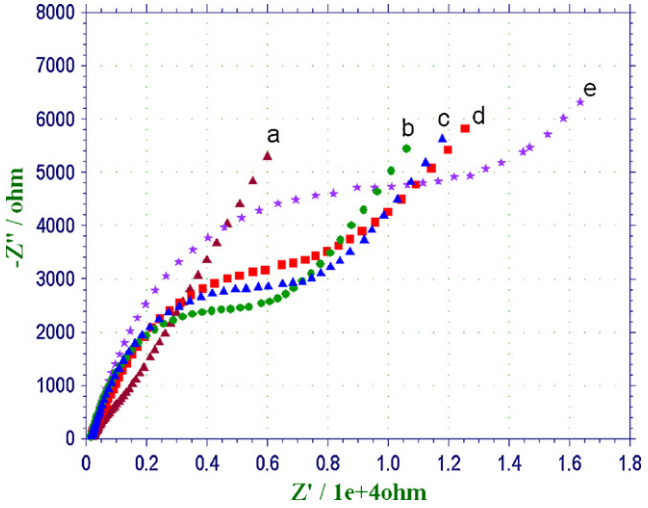

Fig. 4. EIS for bare gold electrode (a), MPTMS–GPTMS/Au (b), S_1/MPTMS–GPTMS/Au (c), Co–S_1–S_2/MPTMS–GPTMS/Au (d) and S_1–S_2/MPTMS–GPTMS/Au (e) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$.

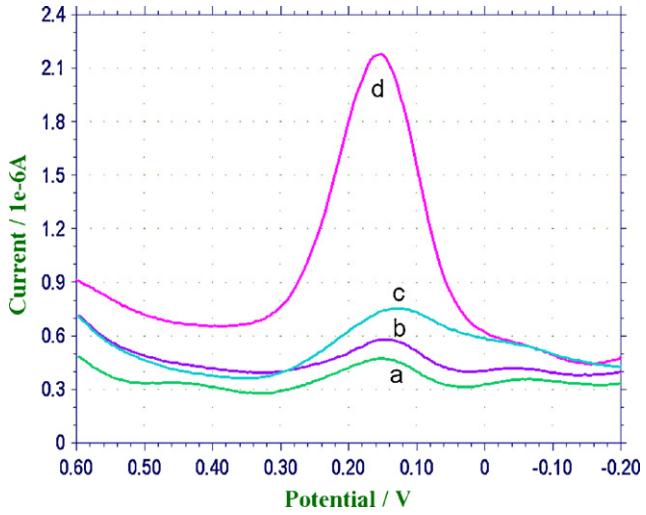

Fig. 5. DPVs of $[Co(phen)_3]^{3+}$ on MPTMS–GPTMS/Au (a), S_1/MPTMS–GPTMS/Au (b), S_1–S_3/MPTMS–GPTMS/Au (c) and S_1–S_2/MPTMS–GPTMS/Au (d) in 0.1 M PBS (pH 6.5). The concentration of S_2 and S_3 was 3.013×10^{-7} and 3.039×10^{-7} M, respectively.

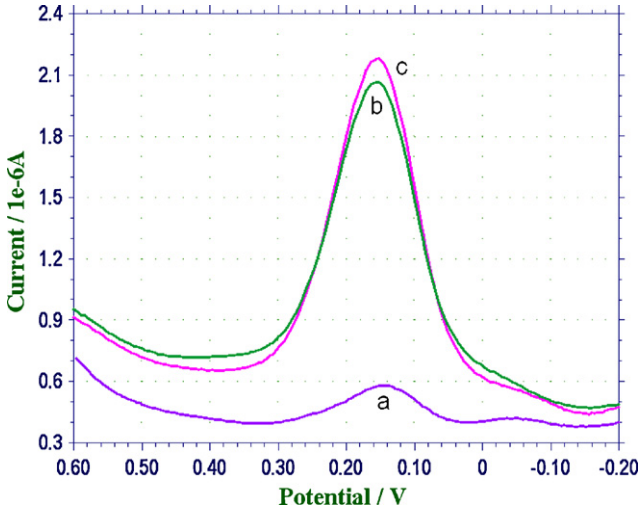

Fig. 6. DPVs of $[Co(phen)_3]^{3+}$ on a DNA probe regeneration/hybridization cycle in 0.1 M PBS (pH 6.5) including the original ssDNA probe (a), 12th regeneration of the DNA probe (b) and hybridization (c).

sol–gel assembled on the surface of Au electrode, the value of R_{et} was found to be 3125 Ω, implying remarkable increase of the resistance to electron transfer of the electrochemical probe (Fig. 4b). When ssDNA was covalently incorporated into the sol–gel film, the R_{et} of S_1/MPTMS–GPTMS/Au electrode was 3338 Ω (Fig. 4c). After S_1–S_2 hybridization, the S_1–S_2/MPTMS–GPTMS/Au electrode exhibited a R_{et} of 5058 Ω (Fig. 4e). When the electrochemical indicator $[Co(phen)_3]^{3+}$ intercalated into the dsDNA, it was surprised to find that the R_{et} of Co–S_1–S_2/MPTMS–GPTMS/Au decreased significantly and a 1901 Ω was evaluated (Fig. 4d). On the basis of the EIS results, we can conclude that NH_2–ssDNA was successfully immobilized on Au electrode via self-assembly of MPTMS–GPTMS sol–gel.

3.4. The selectivity of the developed biosensor and the determination of target ssDNA

The selectivity of the developed DNA electrochemical biosensor was investigated by using the S_1/MPTMS–GPTMS/Au electrode to hybridize with different kinds of DNA sequences including complementary ssDNA sequence (S_2) and non-complementary ssDNA sequence (S_3). Fig. 5 shows the DPV signals of $[Co(phen)_3]^{3+}$ on S_1/MPTMS–GPTMS/Au electrode (Fig. 5b) and after hybridization with the same amount of complementary S_2 (Fig. 5c) or non-complementary DNA sequence S_3 (Fig. 5d). The DPV signal on MPTMS–GPTMS/Au electrode was also compared (Fig. 5a). A high signal was obtained with the S_1–S_2/MPTMS–GPTMS/Au electrode. No changes in signal were observed after S_1/MPTMS–GPTMS/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 DPVs for different concentrations of DNA were investigated (see supplementary material). The average peak current has a good linear relation versus concentration of S_2 in the range of 2.51×10^{-9} to 5.02×10^{-7} M, with a correlation coefficient of 0.9985. The regression equation is $\Delta i_p = 3.968C + 7.162 \times 10^{-8}$ (C, M; Δi_p, A). A detection limit of 8.57×10^{-10} M of the target ssDNA 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; X.M. Li et al., 2007; Li et al., 2008). It might be ascribed to the covalent immobilization of the probe ssDNA.

3.5. Regeneration of the developed biosensor

The S_1–S_2/MPTMS–GPTMS/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 ice 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. 6. After 12 regeneration and hybridization, the electrode possessed 91% of its original current response (Fig. 6b). Therefore, the regeneration of the S_1/MPTMS–GPTMS/Au possessed potential for continuous monitoring of the target DNA in the future.

4. Conclusion

The successful preparation of the DNA electrochemical biosensor demonstrated the efficiency of the covalent immobilization of probe ssDNA via direct coupling of sol–gel and self-assembled technologies. Using epoxide–siloxane and SH–siloxane, the fabrication process offered simple and convenient methodology for self-assembly of functional sol–gel film 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.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Nos. 20775039 and 20775038), the program for New Century Excellent Talents in Universities (No. NCET-04-0649) and the Special Project of Qingdao for Leadership of Science and Technology (No. 05-2-JC-80).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.06.052.

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