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Direct electrochemical sensor for label-free DNA detection based on zero current potentiometry

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ABSTRACT

A direct electrochemical DNA biosensor based on zero current potentiometry was fabricated by immobilization of ssDNA onto gold nanoparticles (AuNPs) coated pencil graphite electrode (PGE). One ssDNA/AuNPs/PGE was connected in series between clips of working and counter electrodes of a potentiostat, and then immersed into the solution together with a reference electrode, establishing a novel DNA biosensor for specific DNA detection. The variation of zero current potential difference (ΔE_{zcp}) before and after hybridization of the self-assembled probe DNA with the target DNA was used as a signal to characterize and quantify the target DNA sequence. The whole DNA biosensor fabrication process was characterized by cyclic voltammetry and electrochemical impedance spectroscopy with the use of ferricyanide as an electrochemical redox indicator. Under the optimized conditions, ΔE_{zcp} was linear with the concentrations of the complementary target DNA in the range from 10 nM to 1 μM, with a detection limit of 6.9 nM. The DNA biosensor showed a good reproducibility and selectivity. Prepared DNA biosensor is facile and sensitive, and it eliminates the need of using exogenous reagents to monitor the oligonucleotides hybridization.

1. Introduction

Nowadays, sensitive and selective DNA detection has become increasingly important in diagnosis, gene therapy and other biomedical studies. In this regard, many techniques including fluorescence (Dragan et al., 2010), quartz crystal microbalance (Kleo et al., 2011; Patolsky et al., 2000), electrochemiluminescence (Lee et al., 2007; Shen et al., 2012), surface plasmon resonance spectroscopy (Moon et al., 2012; Wang et al., 2007) and electrochemistry (Won et al., 2011) have been developed for DNA detection. Among them, electrochemical techniques have attracted considerable attention due to its high sensitivity, rapid response, simplicity, and low cost (Reisberg et al., 2008; Wang et al., 2010, 2011).

One of the key drawbacks of most electrochemical DNA biosensor concepts is the requirement for a labeling step to transduce the hybridization event (Marques et al., 2009; Shiddiky et al., 2010). Although labeling steps can enhance the sensor sensitivity, they increase the time, complexity and cost of the measurement, and perhaps could affect the bioaffinity of the probe. As a result, increasing efforts were devoted to develop a label-free electrochemical DNA biosensor array (Meric et al., 2002; Özkumur et al., 2010; Kerman et al., 2003). Tedious labeling procedures can be avoided by this method, but adscititious signal indicators such as methylene blue (MB) (Siddiquee et al., 2010; Patel et al., 2010), $Ru(NH_3)_6^{3+}$ (Liu et al., 2010a; Wang et al., 2011), $Co(phen)_3^{3+}$ (Li et al., 2010; Liu et al., 2010b), Au nanoparticles (Fan et al., 2011) etc., are often still required. Therefore, a direct detection assay would be preferable (Wu et al., 2010) since such a protocol would greatly simplify the sensing protocol and offer an instantaneous detection of the duplex formation.

An electrochemical method, named zero current potentiometry which was newly developed by our group, has been successfully applied to monitor acid-base property of the carboxylic multi-walled carbon nanotubes (Gao and Song, 2009a) and to develop a polyaniline-based pH sensor (Gao and Song, 2009b). Zero current potentiometry is based on the change in interfacial potential due to the interfacial reaction between substances to be studied at the conducting material/solution interface rather than the change in their redox potential or current (Guo et al., 2011). As a consequence of this feature, it is expected that zero current potentiometry would have the potential for monitoring DNA hybridization without the use of any external reagent or modification of the target. In our strategy, the variation of interfacial potential, upon hybridization of the probe with the target DNA,

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can be directly converted into the measureable response of zero current potential, which opens up a possibility for developing a direct biosensor for DNA detection.

Herein, a direct electrochemical DNA biosensor based on zero current potentiometry, was initially established for DNA sensing in which the hybridization of surface immobilized probe DNA with target complementary DNA can be specifically and quantitatively detected. This new protocol offers cost-effective sensing of DNA and eliminates the necessity of complicated labeling procedures. As established DNA biosensor may have great potential in the detection of target DNA in real samples and the electrode modification strategy is expected to be further applied in protein and enzyme biosensors.

2. Materials and methods

2.1. Reagents

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) was obtained from Aladdin Reagent Ltd., China. 6-Mercapto-1-hexanol (MCH) was obtained from Aldrich Chemical Company. All synthetic oligonucleotides were purchased from Sangon Biotechnology Inc., (shanghai, China). Their base sequences are listed as follows:

Probe DNA: 5′-SH-TTT TTA GAC ATG CCC AGA CAT GCC C-3′
Complementary target DNA: 5′-GGG CAT GTC TGG GCA TGT CT-3′
One base-mismatched DNA: 5′-GGG CAC GTC TGG GCA TGT CT-3′
Three base-mismatched DNA: 5′-GGG CTC TTC TGG GCA TGT CT-3′
Non-complementary DNA: 5′-ACA ACG CCA GAA CAC GAG AG-3′

All oligonucleotide stock solutions were prepared using TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and kept frozen. Diluted solutions were prepared in 50 mM Tris–HCl and 20 mM NaCl buffer solution (pH 7.2). The hybridization solution was diluted with 2 × SSC (pH 7.0), which consists of 0.3 M NaCl and 30 mM sodium citrate buffer solution. All other reagents were of analytical grade and Millipore Milli-Q water (18.2 MΩ) was used throughout.

2.2. Apparatus

All electrochemical measurements were performed on a CHI 660D electrochemical workstation (Shanghai CH Instrument Company, China). The cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a three-electrode system consisting of the modified pencil graphite electrode (PGE) as working electrode, a saturated calomel reference electrode (SCE) and a platinum wire counter electrode. The zero current potentiometry was carried out with a novel electrochemical measurement system. As shown in Fig. 1, a modified PGE was connected in series between the terminal joints of the working electrode and the counter electrode, and then immersed into the solution along with a SCE as the reference electrode, constructing a new electrochemical measurement system. The pencil leads (black lead of degree 2B) were purchased from a local store, China. All leads had a diameter of 0.7 mm.

2.3. Preparation of gold nanoparticles (AuNPs) onto PGEs

Before the gold layer deposition, these PGEs were polished successively with 0.3 μm and 0.05 μm alumina slurry on microcloth pads, subsequently by ultrasonically rinsed with ultrapure water, followed by cycling in 1 M H_2SO_4 until a stable CV was obtained and then rinsed thoroughly with Milli-Q water. The gold

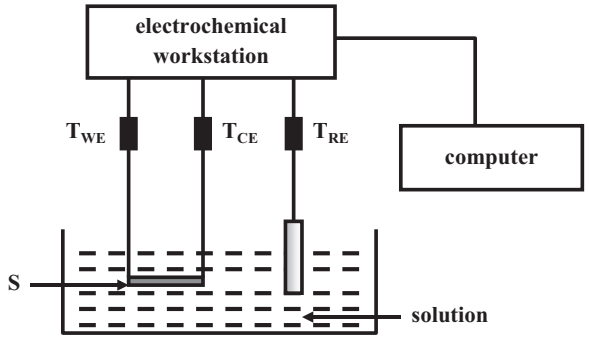

Fig. 1. Schematic illustration of the new electrochemical measurement system. T_{WE}, T_{CE} and T_{RE} are the terminal joints of working electrode, counter electrode and reference electrode of CHI 660D workstation, respectively. *S* is the modified PGE.

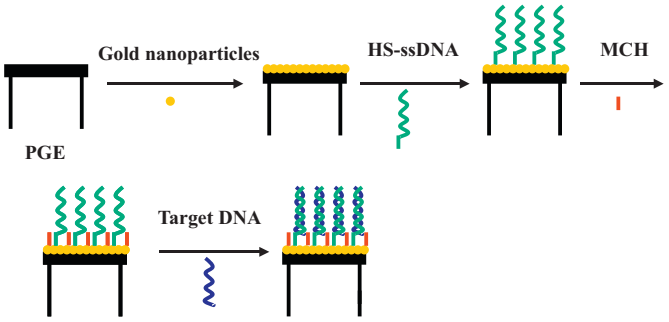

Fig. 2. Schematic illustration of the fabrication processes of DNA biosensor.

nanoparticles electro-deposition on the pretreated electrodes was conducted at −0.25 V (vs. SCE) in a stationary 2 mM $HAuCl_4$ and 0.01 M KCl solution for 60 s. The AuNPs/PGE was washed thoroughly with Milli-Q water for further use.

2.4. Immobilization of probe DNA on gold-coated PGE

The AuNPs/PGE was immersed in 1 mL of 1 μM probe oligonucleotide solution for 10 h at room temperature to obtain a probe DNA modified electrode, followed by extensive rinsing with washing solution (50 mM Tris–HCl+20 mM NaCl, pH 7.2) and Milli-Q water for 5 min to remove unbound probe DNA. Then the electrode was dipped in 1 mM aqueous MCH at room temperature for 30 min to block the unassembled surfaces, and rinsed with washing solution and Milli-Q water thoroughly.

2.5. DNA hybridization

The hybridization was conducted as follows: the probe DNA modified electrode (ssDNA/AuNPs/PGE) was immersed into 2 × SSC buffer solution (pH 7.0) containing the target DNA at 50 °C for 1 h. After hybridization, the electrode was rinsed with washing solution and Milli-Q water for 5 min to remove the non-hybridized DNA. The resulting electrode was denoted as dsDNA/AuNPs/PGE.

The detailed fabrication processes of electrochemical DNA biosensor are schematically illustrated in Fig. 2.

2.6. Electrochemical measurement

The CV measurements were performed in 1 mM $[Fe(CN)_6^{3-/4-}]$ solution containing 0.1 M KCl at the scan rate of 100 mV s^{-1}. The EIS measurements were performed in 10 mM $[Fe(CN)_6^{3-/4-}]$

solution containing 0.1 M KCl with the frequency ranged from 0.1 Hz to 100 kHz.

The zero current potential (E_{zcp}) measurements were carried out with the novel electrochemical measurement system. E_{zcp} was obtained from *I–E* curves of different modified PGEs via linear sweep voltammetry (LSV) by setting the potential between 0.40 and −0.60 V at a scan rate of 100 mV s^{-1}.

3. Results and discussion

3.1. Establishment and characterization of DNA electrochemical biosensor

Pencil graphite, a porous composite comprises three main components: graphite particles, polymeric binder and other additives, hence it is expected that as a composite it should become a good substrate electrode. The usage of pencil graphite lead as substrate electrode has been reported in many applications by virtue of their good mechanical rigidity, low cost, ease of modification, renewal and miniaturization (Isa and Ghani, 2009; Bakhtiarzadeh and Ghani, 2010; Tehrani and Ghani, 2010). However, direct adsorption-based immobilization of bioactive molecules would suffer from unfavorable stability and poor regenerability of sensors (Zhang et al., 2009). Herein, an electro-deposition method was adopted to prepare gold-capped pencil graphite surface. In this case, probe DNA can be immobilized onto the so produced electrode effectively.

The electron transfer of ferricyanide through the modified electrode could be used as a valuable tool to provide some important information about the modified electrode surface. Fig. 1A shows the cyclic voltammograms obtained for different modified electrodes in 1 mM $[Fe(CN)_6^{3-/4-}]$ solution containing 0.1 M KCl at the scan rate of 100 mV s^{-1}. There was no obvious redox peaks of $[Fe(CN)_6^{3-/4-}]$ observed on the bare PGE (curve a). It could be owing to the low electron transfer ability of the bare PGE. After the electrodeposition of AuNPs, a pair of well-defined redox peaks was observed with the anodic (E_{pa}) and cathodic (E_{pc}) peak potential of 0.32 V and 0.16 V, respectively, and a peak potential difference (ΔE_p) of 160 mV (curve b). This could be attributed to the improved electron transfer ability of $[Fe(CN)_6^{3-/4-}]$ by AuNPs modification. Probe DNA immobilized electrode witnessed decreased the peak current of $[Fe(CN)_6^{3-/4-}]$ and increased the ΔE_p (curve c), indicating the probe DNA has been successfully immobilized on the electrode surface and the repellence of $[Fe(CN)_6^{3-/4-}]$ is increased by the negatively charged phosphate backbone of probe DNA (Li et al., 2011). When the hybridization of probe DNA with complementary target DNA proceeded, a further decreased peak current and increased ΔE_p were observed (curve d). Due to the increased repellence of species, duplex formation results in negative charges enhancement on the electrode surface (Wang et al., 2011).

EIS was employed to provide further information on the impedance changes of the electrode surface during stepwise modification. For EIS measurements, $[Fe(CN)_6^{3-/4-}]$ was employed as the redox-probe and Nyquist polts were used to calculate the R_{et} for modified electrodes. As shown in Fig. 1B, bare PGE possessed a remarkably large R_{et} (curve a), indicating slower electron-transfer kinetics of $[Fe(CN)_6^{3-/4-}]$ on bare electrode surface. After AuNPs modification, the R_{et} value decreased to about 1912.7 Ω (curve b), suggesting that the electron transfer ability of $[Fe(CN)_6^{3-/4-}]$ was significantly improved. However, the successive increase in the R_{et} was observed with the self-assembly of probe DNA on the electrode surface (curve c), and thereafter hybridization with complementary target DNA (curve d), with R_{et} values were 2948.1 Ω and 3421.8 Ω, respectively. Such a phenomenon could be attributed to the increased negatively charged phosphate skeletons of DNA on the surface of the electrode that perhaps prevents $[Fe(CN)_6^{3-/4-}]$ ions from reaching the electrode surface for electron-transfer during redox reaction (Ensafi et al., 2011). This reveals that modification and hybridization were successfully accomplished. The impedance experiments could be well corroborated together with the CV experiments to characterize the electrode fabrication process.

3.2. Optimization of the DNA biosensor

The efficiency of the hybridization, which affects the variation of the zero current potential, was optimized in this study with respect to hybridization temperature and hybridization time. The probe DNA modified electrode was immersed in a hybridization solution containing 1 μM target DNA at different temperatures (30 °C, 40 °C, 50 °C, 60 °C and 70 °C). The ΔE_{zcp} before and after hybridization reached the maximum value at the hybridization temperature of 50 °C (see Fig. 2). Thus, 50 °C was chosen as the hybridization temperature at the following experiments. With the hybridization time changing from 40 min to 60 min, 90 min and 120 min, the ΔE_{zcp} almost reached a platform after 60 min (see Fig. 3). Thus, 60 min was selected as the optimized hybridization time.

3.3. Diagnostic performance of the DNA biosensor

Under the optimal experimental conditions, the electrochemical hybridization assay was further investigated by varying the

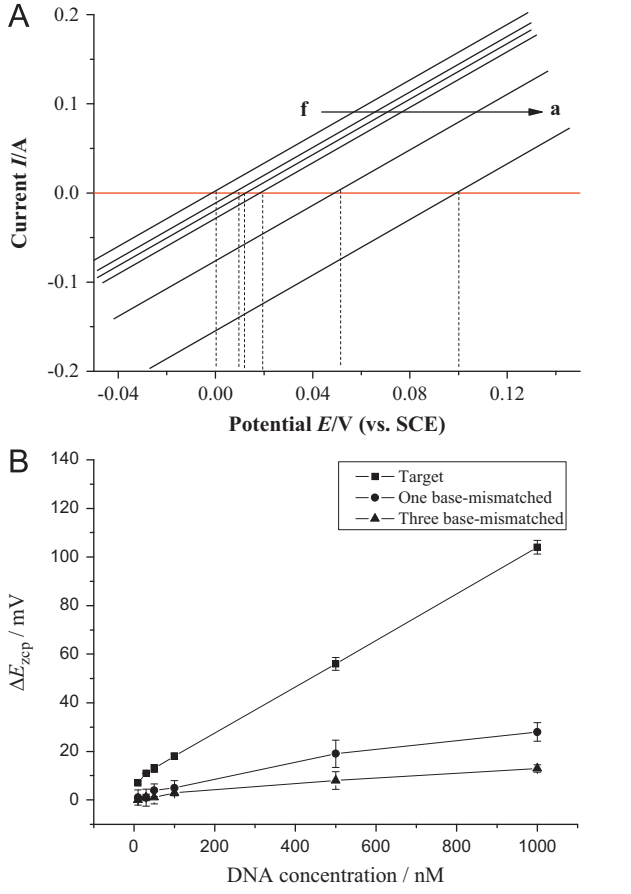

Fig. 3. (A) Linear sweep voltammetry response curves of the biosensor at various target DNA concentration of 10 nM (a), 30 nM(b), 50 nM (c), 100 nM(d), 500 nM(e), 1000 nM(f). (B)The relation of zero current potential difference (ΔE_{zcp}) with target DNA, one base-mismatched DNA and three base-mismatched DNA concentrations ranged from 10 nM to 1 μM. Error bars are the standard deviation of three repetitive experiments.

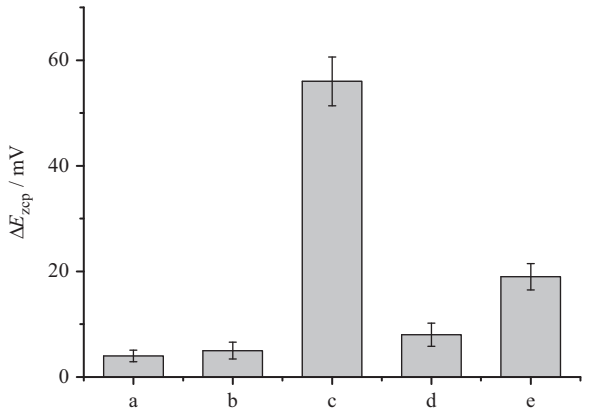

Fig. 4. Bar chart of the ΔE_{zcp} for ssDNA/AuNPs/PGE after the hybridization with blank solution (2 × SSC, pH7.0) (a), 500 nM non-complementary DNA (b), 500 nM complementary target DNA(c), 500 nM three base-mismatched DNA (d) and 500 nM one base-mismatched DNA (e), respectively.

target DNA concentration. The results showed that E_{zcp} shifted negatively in parallel along the potential axis with the target DNA concentration increased from 10 nM to 1 μM (sensitivity is 1 mV) (Fig. 3A). Calibration plots for the target, one base-mismatched and three base-mismatched sequences are shown in Fig. 3B. According to the plot, the mismatched sequences produced at least 60% lower analytical signal and lower correlation coefficient in comparison with the full matching oligonucleotide, indicating that this DNA biosensor is able to detect the perfectly matched complementary sequences sensitively and selectively. ΔE_{zcp} was found to be linear with the concentration of target DNA in the range of 10 nM to 1 μM with a correlation coefficient of 0.9993. The linear regression equation for the target DNA using regenerated electrode was calculated to be $\Delta E_{zcp}=0.09649c+7.65625$ (c was the concentration of target DNA) and the low detection limit of 6.9 nM was estimated as 3σ (where σ is the relative standard deviation of a blank solution, $n=11$). Quintuplicate measurements for the 500 nM target DNA from different electrodes prepared independently were also carried out by the aforementioned conditions, yielding reproducible responses with the relative standard deviation of 2.6%.

Compared with the published researches on direct DNA sensing, the current strategy provides a platform to study DNA hybridization with sensitivity higher or comparable to those reported based on conducting polymer (Cai et al., 2003), sub-optimum displacement (Mir and Katakis, 2008) or monolayer (Zhang et al., 2012a). Although the current strategy is still less sensitive compared with some direct DNA sensors (Zhang et al., 2012b; Reisberg et al., 2006; Wu et al., 2010; Fan et al., 2003; Komarova et al., 2005), it offers an entirely new concept of monitoring DNA hybridization with a rapid response time (s) and does not need reagent-intensive amplification procedures, which is unsurpassed by most sensors of comparable selectivity and sensitivity. In addition, the sensing strategy is of relative simplicity, low cost and satisfactory sensitivity.

3.4. The specificity, regeneration and stability of the fabricated DNA biosensor

The specificity of this DNA biosensor was investigated by control hybridization experiments for blank solution (2 × SSC, pH 7.0), non-complementary DNA, complementary DNA, one base-mismatched DNA and three base-mismatched DNA at 500 nM concentrations of DNA. Because signal generation is based on a hybridization-linked interface potential change, this DNA biosensor should be largely impervious to false positive and negative responses. Consistent with this claim, we find that the complementary target DNA showed a maximal ΔE_{zcp} (Fig. 4c), suggesting that hybridization was formed at the electrode. The non-complementary DNA produced a neglectable ΔE_{zcp} (Fig. 4b), which was similar to that of the background (Fig. 4a), indicating that the hybridization reaction was not achieved. After the ssDNA was hybridized with one base-mismatched DNA sequence (e) or three base-mismatched DNA sequence (d), ΔE_{zcp} was much smaller than that obtained from the hybridization with the complementary target DNA. Random oligonucleotides can be easily discriminated from complementary target DNA via comparing the ΔE_{zcp} value. The results demonstrated that this DNA biosensor displayed very high selectivity for the hybridization detection.

Regeneration is one measure of the sensor's performance, which is important for the continuous detection of the target DNA. Regeneration of dsDNA/AuNPs/PGE was performed at −1.5 V for 5 min to break the gold–sulfur bonds which could allow all the modifiers to be reductively desorbed from the surface of gold layers (Weisshaar et al., 1992). The renewed biosensor can thereby be employed for another round of DNA detection after another preparation. It was found that the fabricated DNA biosensor could be regenerated over ten times with the relative standard deviation within 2.9%. In addition, the stability of the biosensor was investigated, and the experimental data demonstrated that the decrease in the zero current potential for 500 nM target DNA was about 4% after stored at 4 °C for two months.

3.5. Extension to the other synthetic oligonucleotides

See Supplementary material for details.

4. Conclusion

The protocol described here offers an entirely new concept of the development of direct electrochemical DNA biosensor based on zero current potentiometry. The developed DNA biosensor has high selectivity to detect complementary target DNA. It is discovered that signal generation is coupled to a hybridization-linked interface potential change, the duplex formation can be detected by directly measuring interface potential dependent zero current potential. The interface potential will change due to the interfacial reaction, therefore, this method can be extended to develop other sensors. Further researches will be needed to improve particularly the sensitivity and detection time of this method. It is expected that future improvements will accelerate the adaptation of this biosensor in DNA damage analysis and clinical diagnostics.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.07.038.

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