An Electropolymerized Membrane Biosensor for Specific DNA Recognition

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A sensitive electrochemical biosensor for detecting the sequence of short DNA oligomers is represented. The biosensor is based on a platinum electrode covered a polymerized membrane of conductive monomer *N*-[6-(thien-3-yl)acetoxy]-pyrrolidine-2, 5-dione (TAPD). The membrane of TAPD immobilizes a probe DNA on the electrode. The hybridization of the probe with a sequence-specific DNA in sample solutions is monitored by a self-synthesized electroactive indicator, which specifically intercalates in the hybrids on the electrode surface. The current signal of the biosensor is proportional to the concentration of the target DNA in samples, and a very low detection limit of 5×10^{-10} mol/L is found. The biosensor has been used to detect the short oligomers containing of HIV-1 and mycobacterrium nucleotide sequences.

Keywords biosensor, electrochemical polymerization, DNA sequence, hybridization

Introduction

When a ssDNA encounters a complementary partner in a sample solution, it will hybridize. This hybridization can be detected by changes in an electrochemical signal voltage or current. An electrochemical biosensor with the selectivity of biochemical reaction and the sensitivity of electrochemical detection is expected to be a fast, accurate, convenient and low-cost assay for DNA. Currently, some methods based on electrochemical changes during hybridization are reported, and a prototype handheld DNA sensor has been developed.[1-3] Mikkelsen *et al*. reported a DNA sensor based on hybridization indicators. A probe DNA was covalently immobilized onto the carboxylic acid groups on the surfaces of oxidized glassy carbon electrodes, using water-soluble carbodiimide and *N*-hydroxysuccinimide coupling reagents. Hybridization and denaturation of the immobilized probe were detected voltammetrically using $Co(bpy)_3^{3+}$ and $Co(phen)_3^{3+}$.[4] Hashimoto *et al*. devised a kind of electrochemical DNA sensors with graphite electrodes and intercalators.[5] In one of their sensors, a DNA probe with a mercaptohexyl group at the 5′-phosphate end was immobilized on a gold electrode. An electrochemically active dye-Hoechst 33258 was used as an intercalator for voltammetrical detection of dsDNAs formed on the electrode surface.[6] Takenaka *et al*. tested a naphthalene diimide treading intercalator carrying ferrocenyl moieties as its termini, that can discriminate dsDNAs from single stranded counterparts.[7] They used this compound as the electrochemically active ligand on a probe-modified electrode for DNA sensor.[8] Ihara *et al*. developed another electrochemical method using a redactactive ferrocene-modified oligonucleotide and an oligonucleotide anchored on a gold electrode, both of which formed a sandwich-type ternary complex with the target DNA to give an electrochemical response in voltammetry.[9] Wang *et al*. used peptide nucleic acids (PNA) as surface recognition layers of DNA sensor. This kind of sensors

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showed advantages including higher sensitivity and specificity, faster hybridization, minimal dependence on ionic strength and use of shorter probes.[10]

Some conductive reagents for electrochemical biosensors, which can immobilize most of bio-elements such as DNAs, antibodies, enzymes and receptors on electrodes, have been developed in our laboratory.[11,12] In this paper, a conductive compound (TAPD), which could link the compounds with amino groups, was synthesized and electrochemically polymerized on platinum electrodes. The probe DNAs with amino groups were then immobilized on the electrode surface. After hybridization with target DNAs, an electrochemically active intercalator, di[1-(ferrocene-carbamoylpropyl)-tetrahydropyrazine-4-(propylcarbamoylpyridine)]-phenazine (Fc-CTPPZ), was added to quantify the hybrids. A linear relationship between the electrochemical signal and the concentration of the target DNA was found in differential pulse voltammetry (DPV). The experiment to detect specific DNAs of HIV-1 virus and M. tuberculosis on a biochip was explored.

Experimental

Apparatus

A CHI 660 electrochemical analyzer (CH Instruments, Inc.) connected with a PIII/500 computer was used for all electrochemical determinations. The three-electrode system contained of an Ag/AgCl reference electrode, a platinum wire auxiliary electrode and a platinum electrode or a plastic chip electrode with strips of gold film. A NaCl (0.1 mol/L)/Ag/AgCl reference electrode was used in a aqueous solution, and a $AgNO_3$ (0.01 mol/L), tetrabutylammonium fluoroborate (TBAFB) (0.1 mol/L)/actonitrile Ag/AgCl reference electrode was used in a non-aqueous solution.

Reagents

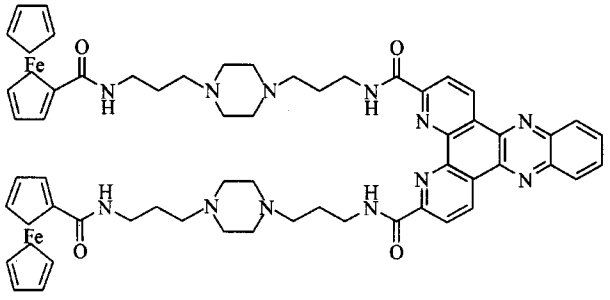
Two probe ssDNAs [5′-ACTGCTAGAGATTTTCCACAT-3′ (pD-1) and 5′-GTCGTCAGACCCAAAACCCCGAGAGGG-3′ (pD-2)] which were complimentary to specific DNA pieces of HIV-1 virus (HIV-1 U5 LTR DNA) and M. tuberculosis (MTB DNA DR),[13,14] two complimentary target ssDNAs [5′-ATGTGGAAAATCTCTAGCAGT-3′ (tD-1) and 5′-CCCTCTCGGGGTTTTGGGTCTGACGAC-3′ (tD-2)] and two non-complimentary ssDNAs [5′-GCTAGGCCAAATGCAGGTTTACGTA-3′ (ncD-1) and 5′-TTAGCTAAGTCG-3′ (ncD-2)] were provided by the Biochemistry Institute, Zhejiang University with a concentration of 2×10^{-3} mol/L. 3-Thiophene acetic acid (TAA) was purchased from Acros, and *N*-hydroxysuccinimide (NHS) and 1-[3-(dimethylamino)propyl]-3-ethylcardodiimide (DPEC) were obtained from Aldrich. A phosphate buffer solution (0.1 mol/L, pH 7.4, PBS) and all other solutions were prepared with double-distilled water.

Synthesis

N-[6-(*thien*-3-*yl*) *acetoxy*]-*pyrrolidine*-2, 5-*dione* (TAPD) 2.8 g of TAA in a stirring chloroform solution was reacted with 2.3 g of NHS and DPEC for 5 h at room temperature. 2.7 g of brown oil was obtained after chromatographic purification with a column of silica gel (Merck 60; chloroform : methanol = 4 : 1, *V*: *V*). The product was identified to be TAPD with a molecular weight of 239 by MS spectra. ^{1}H NMR spectra were recorded on a BRUKER DMX-400 in $CDCl_3$. Chemical shifts were recorded in δ: 2.60—3.00 (m, 4H), 3.62—3.66 (m, 2H), 7.28—7.34 (m, 1H), 7.64—7.68 (m, 1H), 7.85—7.92 (m, 1H). The theoretical contents of elements were calculated for TAPD: C 50.21%, H 3.77%, N 5.68%. Analytical results were found: C 50.67%, H 4.02%, N 5.41%. The oil was dissolved in 40 mL of acetonitrile with a concentration of 0.28 mol/L.

Di[1-(*ferrocene-carbamoylpropyl*)-*tetrahydropyrazine*-4-(*propylcarbamoyl-pyridine*)]-*phenazine* (Fc-CTPPZ) Dipyrido[3,2-a:2′,3′-c]phenazine-3,6-dicarboxylic acid ($\mathbf{A_1}$) was prepared according to the reference.[15] $\mathbf{A_1}$ was activated with DPEC and NHS, then reacted with excess *N*, *N*′-bis(3-aminopropyl)pipoazine in tertrahydrofuran (THF) at room temperature for 5 h. A brown product ($\mathbf{E_1}$) was obtained after working up and recrystallization from ether. $\mathbf{E_1}$ and the NHS ester of ferrocenecarboxylic acid were stirring in chloroform at room temperature for 48 h. The solvent was removed and the residue was separated in a column of silica gel (Merck 60, methanol). Then the solvent was removed under reduced pressure and the residue was dissolved in a small amount of chloroform. The solution was poured into ether and a residue was obtained by filtration. The solid residue

was dissolved in methanol and the resultant solution was poured into water. A dark brown product (Fc-CTPPZ) was separated by filtration and purified by recrystallization from acetone. ^{1}H NMR spectra were recorded on a BRUKER DMX-400 in $CDCl_3$. Chemical shifts were recorded in δ: 1.57—1.61 (m, 4H), 1.69—1.73 (m, 4H), 2.36—2.48 (m, 12H), 2.50—2.60 (m, 12H), 3.43—3.47 (m, 4H), 3.71—3.75 (m, 4H), 4.30—4.70 (m, 18H), 7.05—7.15 (br, 4H), 7.75—7.80 (m, 2H), 8.24—8.28 (m, 2H), 8.60—8.75 (m, 4H). The theoretical contents of elements were calculated for Fc-CTPPZ: C 64.25%, H 6.05%, N 14.51%. Analytical results were found: C 64.41%, H 6.38%, N 14.18%. A stock solution was prepared in an acetonitrile/NaCl (0.1 mol/L) solution (1:1). The molecular structure of Fc-CTPPZ is as follows:



Preparation of DNA *probe electrodes*

The platinum electrode surface was polished with alumina powder, and then washed by water and acetonitrile. The cleaned electrode was immersed in a TBAFB (0.1 mol/L)/actonitrile solution with TAPD (5×10^{-2} mol/L) for cyclic voltammetry (CV). The potential scanned form 0 V to 2.0 V in 20 cycles with a scan rate of 100 mV/s. After electrochemical polymerization, a 10 μL of pD-1 or pD-2 solution (2.5×10^{-4} mol/L) was pipetted to the electrode surface covered TAPD film for 30 min at the room temperature. The modified electrode was cleaned in stirring distilled water for 5 min, and moved in a NaCl solution (0.1 mol/L) to examine the blank current.

Measurement procedure

The DNA probe electrode was immersed in a DNA target solution (0.1 mol/L phosphate and 0.1 mol/L NaCl, pH 7.4) at 80 ℃ for 30 min. After hybridization, the electrode was sequentially washed to remove DNSs bound non-specifically. Then, the electrode was soaked in a stirring Fc-CTPPZ solution for 10 min for intercalation and washed by distilled water. The electrode with hybrids and the intercalator was connected with CHI 660 for electrochemical determination at 25 ℃ in a NaCl solution (0.1 mol/L). The potential scanned from 0.2 V to 0.8 V in differential pulse voltammetric measurements (DPV) with a scan rate of 20 mV/s, an amplitude of 50 mV and a pulse repetition of 0.2 s. For amperometry the potential was fixed at 0.6 V.

Results and discussion

Electrochemical polymerization of TAPD *on electrode surface*

The TAPD was polymerized electrochemically on platinum electrodes by 20 cycles of CV from 0 V to 2.0 V. During the experiment, the color of the electrode surface became light brown and a high peak at 1.77 V was observed in the voltammogram (Fig. 1). It suggested that the TAPD monomer had been oxidized to form a polymer film on the electrode surface.

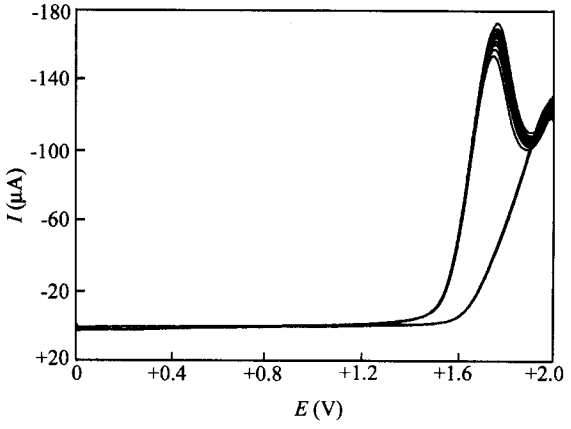


Fig. 1 Cyclic voltammograms of TAPD electropolymerization on a platinum electrode in a TBAFB (0.1 mol/L)/acetonitrile solution with TAPD (5×10^{-2} mol/L).

Cyclic voltammetric determinations were made in a ferrocyanide solution (5×10^{-3} mol/L) with a bare platinum electrode (1) and a TAPD electropolymerized electrode (2). Cyclic voltammograms are illustrated in Fig. 2. Comparing curve 1 and 2, a potential shift of anodic, cathodic peaks ($\triangle E$) and a decrease of peak current are

observed obviously. However, two peaks in curve 2 are symmetric, and there is no sharp decrease on the peak current. It means that the TAPD membrane has been formed on the electrode surface, and the electrode remains good conductivity.

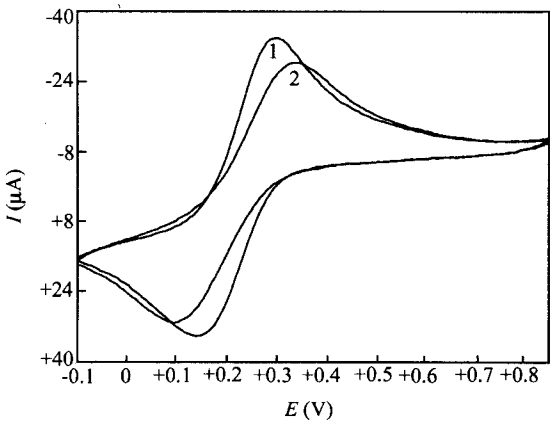


Fig. 2 Cyclic voltammograms of a bare electrode (1) and a TAPD membrane electrode (2) in a ferrocyanide (5×10^{-3} mol/L)/PBS solution.

Immobilization of ssDNA *on* TAPD *membrane*

It is in the condition of room temperature for succinimide group of TAPD to react with an amino group of another compound, and two compounds will be then cross-linked together. In this paper, ssDNAs with amino group on their 5′ end were immobilized by TAPD membrane on electrodes to detect target DNAs in sample solutions. In order to measure the reactivity of TAPD on electrode surface, an electroactive compound with an amino group at its one end and a ferrocene group at another end [1-(Fc-carbamoylpropyl)-4-(aminopropyl)-tetrahydropyrazine, Fc-CAT] was synthesized. There was obvious peak current observed at 0.56 V when a TAPD polymerized electrode, after immersing in a stirring PBS solution contain the Fc-CAT for 10 min, was scanned by potential. It suggests that TAPD has connected Fc-CAT effectively onto the electrode surface. In further experiments, Fc-CAT immobilized on TAPD layer still kept electroactivity under 60 ℃ and 92 ℃, which meant that this cross-linking compound was stable during the temperature variation for DNA hybridization.

Intercalation and electrochemical response of Fc-CTPPZ

If there was no pretreatment of Fc-CTPPZ, both ssDNA electrodes and dsDNA electrodes did not show any current signal during electrochemical measurements. There was a pair of clear current peaks in cyclic voltammograms for dsDNA electrodes after reacting with Fc-CTPPZ (Fig. 3). The potential of the oxidative peak is at 0.51 V and the reductive peak at 0.44 V, due to the intercalation of Fc-CTPPZ to base pairs of DNAs, and ferrocene groups of the molecules are then oxidized during the potential scan. This permits us to estimate that Fc-CTPPZ binds only to dsDNAs, and this compound can be used to recognize the hybridization on electrode surface.

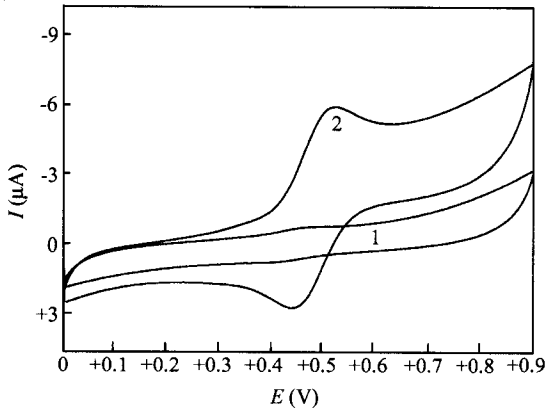


Fig. 3 Cyclic voltammograms of a ssDNA electrode (1) and a dsDNA electrode (2) after the pretreatment of Fc-CTPPZ.

Condition and selectivity of hybridization

DNA hybridization is deeply affected by reaction time and temperature. In this experiment, there was only a poor current response observed when the reaction time was less than 20 min, and the current signal became larger and tended to the maximum after 30 min. It appeared that most probe DNAs on the electrode surface had hybridized with target DNAs after 20 min. The effect of temperature on hybridization is complicated. If the temperature is low, the reaction is difficult to take place. Otherwise, target DNAs will denature when the temperature is high. There was almost no current measured in the experiment when the temperature was lower than 45 ℃, increasing signals were observed at the range of 45—80 ℃, the signal decreased over 80 ℃ (Table 1). So, a reaction time of 30 min and a temperature of 80 ℃ were chosen for the DNA hybridization throughout the experiment.

In order to examine the selectivity of TAPD-DNAs on electrode surface, pD-1 and pD-2 modified electrodes

were challenged with different non-complementary DNA samples (ncD-1 and ncD-2). Results in Table 2 showed that non-complementary DNAs could not react with ssDNAs on electrodes, and almost no electrochemical response was observed. It means that this electrochemical biosensor remains high selectivity of DNA hybridization.

Table 1 Electrochemical response of biosensors at different temperatures[a]

Probe DNA	DNA	Temperature (℃)	Current response (nA)
pD-1	tD-1	25	3
pD-1	tD-1	40	5
pD-1	tD-1	60	67
pD-1	tD-1	80	111
pD-1	tD-1	92	94
pD-2	tD-2	25	6
pD-2	tD-2	40	3
pD-2	tD-2	60	74
pD-2	tD-2	80	128
pD-2	tD-2	92	86

[a] Probe DNA concentration: 4×10^{-8} mol/L; hybridization time: 30 min; hybridization solution: phosphate (0.1 mol/L) and NaCl (0.1 mol/L), pH 7.4. The current responses were observed in DPV experiments in a NaCl solution (0.1 mol/L).

Table 2 Electrochemical response of biosensors in non-complementary DNAs[a]

Probe DNA	DNA	Current response (nA)
pD-1	ncD-1	7
pD-1	ncD-2	3
pD-1	tD-2	4
pD-2	ncD-1	3
pD-2	ncD-2	6
pD-2	tD-1	5

[a] Hybridization temperature: 80 ℃, other conditions are the same as in Table 1.

Calibration curve and detection limit

The utility of the new biosensor depends on achieving well-defined concentration dependence. In DPV experiments, linear calibration plots were obtained within the range of $(0—10) \times 10^{-8}$ mol/L for five successive 2×10^{-8} mol/L increments of the target tD-2 (a slop of 119 nA/10 nmol · L^{-1} and a correlation coefficient of 0.9975). Calibration curve for concentration of target DNA was shown in Fig. 4. A series of five repetitive measurements of 4×10^{-8} mol/L resulted in reproducible signals (473—519 nA) with a relative standard deviation of 8.4%. A detection limit of 5×10^{-10} mol/L for this DNA biosensor was estimated based on the signal to noise characteristics ($S/N = 3$) in the measurement of 1×10^{-8} mol/L of the target tD-2, in which a signal of 118 nA was detected while a mean of noise was 2 nA.

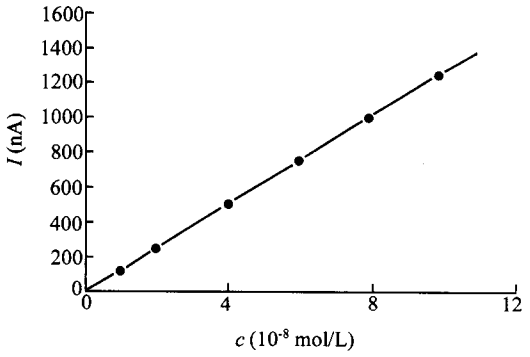


Fig. 4 Calibration curve for concentration of target DNA.

Detection of short DNA *sequences related to* HIV-1 *and M. tuberculosis in chips*

In further experiment, ssDNAs related to HIV-1 and M. tuberculosis were immobilized respectively on two gold strips (1 × 10 mm) in a plastic piece (5 × 10 mm). The plastic electrode was used for detection in a mixed solution with pD-1, pD-2 and ncD, and both of gold strips showed current responses (0.26 μA and 0.24 μA). When the plastic electrode was immersed in a solution contained tD-1 and ncD, there was an obvious current (0.25 μA) only at the strip modified with HIV-1 target DNA (pD-1), comparing with a current of 0.02 μA for another strip modified with M. tuberculosis target DNA (pD-2). It appears that the modified electrodes can be multiplied in a small chip for DNA measurement.

Conclusions

A new conductive monomer TAPD for electrode modification was synthesized, which is an effective and convenient method to electropolymerize TAPD on electrode surface and cross-link probe DNAs with its succinimide group. An intercalator Fc-CTPPZ with two ferrocenyl groups was also synthesized for detecting the electrochemical signal of DNA hybridization. The high sensitivity of the biosensor is attributed to the good conductivity of

TAPD membrane and the high electrochemical activity of Fc-CTPPZ. This TAPD electropolymerized membrane is not only for immobilizing oligonucleotide, but also for linking other biomaterials with amino groups (such as enzymes and antibodies) for enzyme electrodes and immunosensors. It is possible to multiply different biosensors at a microchip to detect various viruses and genes simultaneously for clinic diagnosis.

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