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Direct DNA Hybridization Detection Based on the Oligonucleotide-Functionalized Conductive Polymer

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Electrochemical methods for DNA hybridization detection have many advantages that are very fast to detect hybridization and can be directly applied for a portable DNA sensor. In this paper, an electrochemical method to directly detect DNA hybridization was developed on the basis of a new conductive polymer, which was polymerized on the glassy carbon electrode with a terthiophene monomer having a carboxyl group (3'-carboxyl-5,2',5',2''-terthiophene). The ss-DNA probe was made by chemically bonding an amine-linked C₆ alkyl group to the 5' terminus of oligonucleotide (19-mer). The probe moiety was immobilized on the polymer through covalent bonding with a catalyst, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. A difference in admittance was observed before and after hybridization as a result of the reduction of the resistance after hybridization. The highest difference in admittance was observed around 1 kHz before and after hybridization. Hybridization amounts of end two-base and center one-base mismatched sequences were obtained only in a 14.3% response when compared to that for the complementary matched sequence.

Biotechnology and medical diagnostics are currently in need of devices that are capable of continuously and selectively detecting biological molecules. Recent trends in nucleic acid electrochemistry are focused on the development of electrochemical biodetectors or biosensors based on specific DNA interactions, including DNA hybridization, DNA interactions with drugs or carcinogens, and DNA damage. Of these, diverse DNA sensors have been used to detect DNA sequence,¹ toxic compounds,² and trace organic compounds.³ Detection of hybridization for a DNA sequence is the most dependable approach because of its wide applicability in regard to genomics, determination of relative gene expression, etc. The various methods utilize optical,^{4,5} surface

acoustic wave,⁶ quartz crystal microbalance (QCM),^{7,8} and electrochemical methods.^{9–13} Most of the optical and electrochemical methods are indirect, such as using dyes or redox indicators. Electrochemical methods have been extensively studied for nucleic acids analysis since the electroactivity of nucleic acids was discovered about 40 years ago.¹⁴ The use of metal complex¹⁵ or organic indicators⁹ as markers was extensively studied in regard to hybridization detection using the electrochemical method. However, it is still difficult to develop actual electrochemical devices for automating the process and for making an array chip. To overcome these disadvantages, this study demonstrates the simple electrochemical method for detection of hybridization that completely distinguishes between matched and mismatched sequences. This method employs the difference of the impedance between before and after hybridization for the single-stranded (ss) probe oligonucleotide (ODN; NH₂-C₆-5'-CTCCTGTGGAGAA GTCTGC-3'), which is related to sickle cell anemia, and the target ODN (3'-GAGGACACCTCTTCAGACG-5').

Immobilization of the probe for sequence detection could be promoted by modification with amino, thiol, or biotin groups. Immobilization of the probe on the substrates can be usually achieved using (1) the potentiostatic method,^{1,2} (2) the direct self-assembly method,¹⁶ and (3) the covalent bonding formation method.¹⁷ Garnier et al. introduced a polypyrrole-based probe that detected the hybridization of a target ODN using a potential shift

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- (1) Wang, J.; Cai, X.; Rivas, G.; Shiraishi, H.; Farias, P. A. M.; Dontha, N. *Anal. Chem.* **1996**, *68*, 2629–2634.
- (2) Wang, J.; Rivas, G.; Luo, D.; Cai, X.; Valera, F. S.; Dontha, N. *Anal. Chem.* **1996**, *68*, 4365–4369.
- (3) Oliveira Brett, A. M.; Serrano, S. H. P.; Gutz, I.; La-Scalea, M. A.; Cruz, M. L. *Electroanalysis* **1997**, *9*, 1132–1137.
- (4) Vo-Dinh, T.; Houck, K.; Stokes, D. L. *Anal. Chem.* **1994**, *66*, 3379–3383.

- (5) Chen, X.; Zhang, X. E.; Chai, Y. Q.; Hu, W. P.; Zhang, Z. P.; Zhang, X. M.; Cass, A. E. G. *Biosens. Bioelectron.* **1998**, *13*, 451–458.
- (6) Su, H.; Kallury, K. M. R.; Thompson, M. *Anal. Chem.* **1994**, *66*, 769–777.
- (7) Okahata, Y.; Matsunobu, Y.; Ijio, K.; Mukae, M.; Murakami, A.; Makino, K. *J. Am. Chem. Soc.* **1992**, *114*, 8299–8300.
- (8) Caruso, F.; Furlong, D. N.; Niikura, K.; Okahata, Y. *Colloids Surf. B* **1998**, *10*, 199–204.
- (9) Hashimoto, K.; Ito, K.; Ishimori, Y. *Anal. Chem.* **1994**, *66*, 3830–3833.
- (10) Millan, K. M.; Saraullo, A.; Mikkelsen, S. R. *Anal. Chem.* **1994**, *66*, 2943–2948.
- (11) Takenaka, S.; Yamashita, K.; Takagi, M.; Uto, Y.; Kondo, H. *Anal. Chem.* **2000**, *72*, 1334–1341.
- (12) Millan, K. M.; Mikkelsen, S. R. *Anal. Chem.* **1993**, *65*, 2317–2323.
- (13) Palecek, E.; Fojta, M. *Anal. Chem.* **2001**, *73*, 75A–83A.
- (14) Palecek, E. *Nature* **1960**, *188*, 656–657.
- (15) Carter, M. T.; Bard, A. J. *J. Am. Chem. Soc.* **1987**, *109*, 7258–7530.
- (16) Berggren, C.; Stalhandske, P.; Brundell, J.; Johansson, G. *Electroanalysis* **1999**, *11*, 156–160.
- (17) Korri-Yousseoufi, H.; Garnier, F.; Srivastava, P.; Codillot, P.; Yassar, A. J. *Am. Chem. Soc.* **1997**, *119*, 7388–7389.

and redox current.¹⁷ This is still not satisfactory, however, for the actual application. Thus, in this study, we designed a biosensor using electroactive polyterthiophene functionalized with an ODN probe. Specific hybridization of this grafted ODN with its complementary ODN target in a solution induced a significant change in impedance.

Earlier impedance measurements for DNA and ODNs were introduced by Lawrence et al.¹⁸ They reported the possibility of direct detection of hybridization between complementary homooligomer DNA strands using impedance measurements on functionalized heterostructures. The result shows a curve-plotted Z_q (out-of-phase impedance) vs the applied potential obtained after single-stranded DNA immobilization and then ex-situ hybridization with complementary poly(dA). A curve shift toward negative potentials was systematically obtained after the ex-situ hybridization. Otherwise, in our study, impedance and admittance changes according to frequency variation were observed before and after hybridization.

MATERIALS AND METHODS

Materials. Acetate buffer (pH 5.2, DNase, RNase, and protease, none detected), sodium phosphate dibasic (DNase, RNase, and protease, none detected), sodium chloride (DNase, RNase, and protease, none detected), sodium dihydrogenphosphate (DNase, RNase, and protease, none detected), 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDAC), and dichloromethane (99.8%, anhydrous) were received from Sigma Co. Tetrabutylammonium perchlorate (TBAP, electrochemical grade) was received from Fluka Co. Target ODNs and amino-modified immobilization probes were synthesized by Genemed Synthesis, Inc (South San Francisco, CA 94080). The sequences were immobilized probe (sequence A: $\text{NH}_2\text{-C}_6\text{-5'-CTCCTGTGGAGAAG TCTGC-3'}$), target complementary (sequence B: $3'\text{-GAGGACACCTCTTCAGACG-5'}$), one-base mismatch (sequence C: $3'\text{-GAGGACTCCTCTTCAGACG-5'}$), end two-base mismatch (sequence D: $3'\text{-CTGGACACCTCT TCAGACG-5'}$), noncomplementary (sequence E: $3'\text{-CCTAGTCTACAGGTCACCTA-5'}$). The ODN probe and EDAC solutions were prepared with acetate buffer (30 mM, pH 5.2), and target solutions were prepared with a phosphate buffer solution (pH 7.0) containing 0.75 M NaCl.

A terthiophene monomer bearing a carboxyl group, 3'-carboxyl-5,2',5',2-terthiophene was newly synthesized, which was potentially useful starting material for the synthesis of functionalized polyterthiophenes. It was prepared by the treatment of 3'-bromothiophene with CuCN in DMF followed by hydrolysis in KOH-ethoxyethanol. IR, NMR, and elemental analysis confirmed the product.¹⁹

Differential Pulse Voltammetry and Impedance Measurements. Glassy carbon and ODN probe-modified glassy carbon electrodes (area = 0.07 cm²) were used as working electrodes; a Ag/AgCl (in saturated KCl) electrode, as a reference electrode; and a Pt wire, as a counter electrode. Differential pulse voltammograms were recorded using a Potentiostat/Galvanostat (PAR EG&G model 273A). The impedance spectra were measured with the Potentiostat/Galvanostat and a Lock-In Amplifier (PAR EG&G, model 5210) at a given open circuit voltage from 100 kHz down

to 10 Hz at a sampling rate of 10 points per decade (AC amplitude, 10 mV). A Ag/AgCl (in saturated KCl) reference electrode with a nonaqueous solution bridge was used for the polymer growth in a 0.1 M TBAP/CH₂Cl₂ solution.

Electrochemical Quartz Crystal Microbalance. The experiment employing the electrochemical quartz crystal microbalance (EQCM) was performed using a SEIKO EG&G model QCA917. The EG&G PARC software package (M270/250 Electrochemical Analysis Software) and a Au working electrode (area, 0.196 cm²; 9 MHz; AT-cut quartz crystal) were used for EQCM experiments.

RESULTS AND DISCUSSION

3'-Carboxyl-5,2',5',2-terthiophene was polymerized on a glassy carbon electrode (area, 0.07 cm²) in a 0.1 M Bt₄NClO₄/CH₂Cl₂ solution containing a 1.0 mM monomer by potential cycling five times from 0.0 V to +1.5 V vs. Ag/AgCl. During the first anodic scan from 0.0 V to +1.5 V in a monomer-containing solution, the oxidation peak of the monomer appeared at +1.3 V, and the reverse scan to the positive direction showed a small cathodic peak at +0.9 V, which corresponded to the reduction of the polymer film formed on the electrode. The peak potentials of the polymer itself shifted to a more positive direction as the number of potential cyclings increased. The peak then overlapped with the redox peaks of the monomer oxidation peak, which appeared at around +1.3 V. The resulting blue-colored polymer film showed a broad redox peak around +1.1 V. SEM characterized the polymer film. The morphology of the polymer-coated electrode was homogeneous and porous. SEM assumed the thickness of the polymer film by potential cycling five times about 300 nm.

The thoroughly washed electrode coated with polymer—terthiophene bearing carboxylic acid groups was used to attach the 19-mer probe ODN, as shown in Scheme 1. The polymer-coated electrode was immersed in a 30 mM acetate buffer (pH 5.2) containing 4.0 mM EDAC for 1 h. The EDAC-attached electrode was washed with an acetate buffer solution and subsequently incubated in an 11 μM probe ODN/acetate buffer solution for 6 h at 25 °C. By this process, the amino-linked C₆-modified probe ODN was immobilized on the polyterthiophene film through the formation of covalent bonds with carboxyl groups on the polymer.

The amount of the probe ODN immobilized on the polymer-coated electrode was determined using the QCM technique. The polycarboxyterthiophene-coated electrodes were washed with distilled water and transferred into a QCM cell containing a 100-μL aliquot of EDAC (10 mg/mL) in a 30 mM acetate buffer solution for 1 hr to allow the reaction between the carboxy groups of the polymer and EDAC. After the reaction, the polymer-coated electrode treated with EDAC was washed with distilled water and, finally, reacted in the QCM cell containing 100 μL of a 11 μM probe ODN solution. During immobilization of the probe ODN, the frequency gradually decreased and reached a steady state after 1 h, indicating that the immobilization time was about 1 h. After immobilization for 1 h, the mass change due to immobilization of the probe ODN on the modified electrode surface was calculated to be 352 ± 10 ng as follows:

$$\begin{aligned}\Delta\text{mass} &= \Delta\text{freq} \times 5.608 \text{ (ng/cm}^2\text{)}/\text{Hz} \\ &= 320 \text{ Hz} \times 5.608 \text{ (ng/cm}^2\text{)}/\text{Hz} \\ &\approx 352 \text{ ng}\end{aligned}$$

(18) Souteyrand, E.; Cloarec, J. P.; Martin, J. R.; Wilson, C.; Lawrence, I.; Mikkelsen, S.; Lawrence, M. F. *J. Phys. Chem. B* **1997**, *101*, 2980–2985.

(19) Lee, T. Y.; Shin, S. C.; Shim, Y. B. Submitted.

Scheme 1. Immobilization of the Probe Oligonucleotide and Hybridization for a Target Sequence

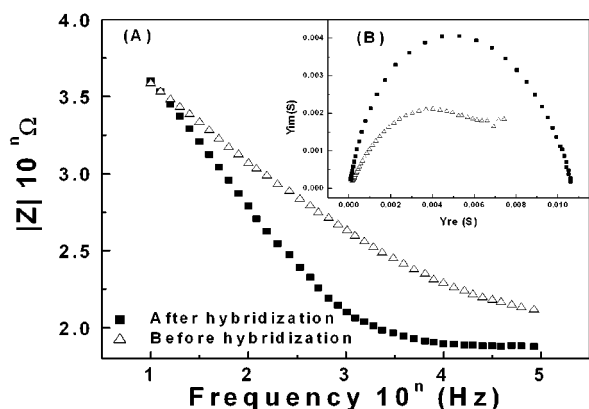
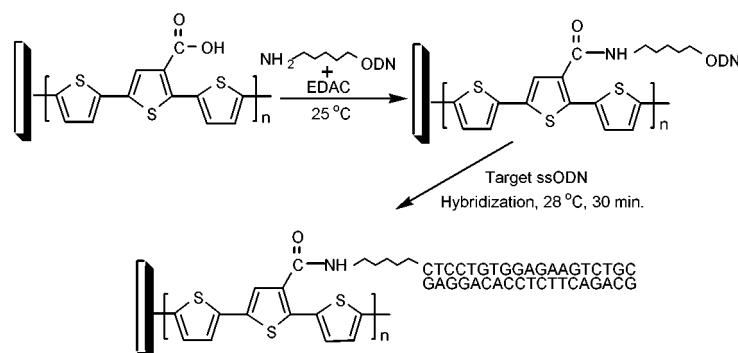


Figure 1. Plots of (A) impedance and (B) admittance before and after hybridization in a phosphate buffer solution on an open circuit voltage at room temperature.

where the Δf_{res} is 320 Hz, and using the physical constant for quartz gives a sensitivity of 5.608 (ng/cm²)/Hz.

To evaluate the immobilized probe, differential pulse voltammograms (pulse height, 50 mV; pulse width, 50 mV; scan rate, 5 mV/sec) were recorded in a blank phosphate buffer (pH 7.0/0.75 M NaCl) solution before and after immobilization of the probe. A voltammogram recorded for polyterthiophene bearing carboxylic acid groups shows a broad anodic peak around +0.95 V. Meanwhile, in the voltammogram recorded for the modified electrode after immobilization with a probe ODN, two peaks appeared at +0.95 V and +1.42 V, which correspond to the oxidation of guanine and adenine on the probe ODN.

This is similar to other reports²⁰ regarding the oxidation potential¹³ of guanine (+0.87 V) and adenine (+1.12 V) of DNA, which were modified on a substrate different from ours.

To hybridize, the probe-immobilized electrode was subsequently incubated for 30 min at 28 °C in a target ODN solution (11 μ M target ODN, phosphate buffer pH 7.0, 0.75 M NaCl). After incubation, this electrode was washed using the same buffer solution. Impedance spectra (frequency range, 100 kHz to \sim 10 Hz; AC amplitude, 10 mV) were obtained for the electrode modified with the probe ODN before and after hybridization reactions. Figure 1 shows plots of (A) impedance and (B) admittance spectra recorded for the probe ODN-modified electrode before and after hybridization with the fully complementary target ODN in a phosphate buffer solution containing 0.75 M NaCl.

Table 1. Hybridization Amounts at 25 °C between the Probe-Immobilized Electrode and Various 19-mer Target Oligonucleotides^a in a Solution at 1 KHz

oligonucleotides sequence ^a	$\Delta Z $ 10 ³ Ω	hybridization ^b %
complementary: 3'-GAGGACACCTCTTCAGACG-5'	0.7 \pm 0.05	100
center one-base mismatch: 3'-GAGGACTCCTCTTCAGACG-5'	0.1 \pm 0.05	14.3
end two-base mismatch: 3'-CTGGACACCTCTTCAGACG-5'	0.1 \pm 0.05	14.3
noncomplementary: 3'-CCTAGTCTACAGGTCACTA-5'	0.0 \pm 0.05	0

^a Concentration, 110 nmol in 10 mL. ^b Indicates how much of the oligonucleotide in the solution is bound to the probe on the modified electrode.

The hybridization causes the decrease of the impedance values, indicating that ds-DNA is more conductive than ss-DNA.

The much higher conductivity of ds-DNA compared to ss-DNA is a well-known phenomenon nowadays.²¹ In this study, the hybridization of the target DNA was easily confirmed by the decrease in impedance, even without an indicator or other label molecules.

The largest reproducible difference of logarithmic impedance values before and after hybridization for the complementary sequence was around 1.0 kHz. However, as shown in Table 1, no difference between those spectra was obtained before and after hybridization with the noncomplementary target ODN (3'-CCTAGTCTACAGGTCACTA-5'). After this experiment, the probe electrode used just before was hybridized with a complementary target ODN. It also gave the largest difference between before and after hybridization at 1.0 kHz. In the evaluation experiment to determine the response to mismatched target ODNs, such as one-base mismatch (3'-GAGGACTCCTCTTCAGACG-5') and end two-base mismatch (3'-CTGGACACCTCTTCAGACG-5'), there was not enough difference of logarithmic impedance values between that before and after hybridization. This means that the probe ODN-modified electrode fabricated in this study specifically discriminated between matched and mismatched ODN sequences. Other previous studies reported that detection of hybridization employing the QCM technique⁷ did not completely discriminate between matched and mismatched sequences. They reported a 92% response for end two-base and a 30% response for center one-base mismatched sequences, respectively, when they were

(20) Cai, X.; Rivas, G.; Farias, P. A. M.; Shiraishi, H.; Wang, J.; Palecek, E. *Electroanalysis* **1996**, 8, 753–758.

(21) Wilson, E. K. *Chem. Eng. News* **1998**, May 25, 47–49.

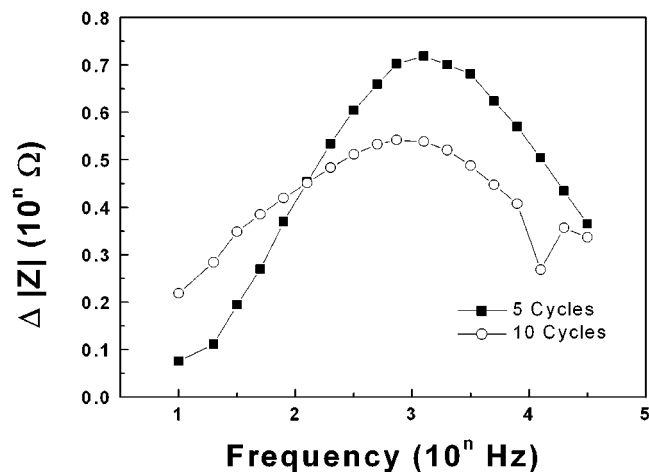


Figure 2. Differences of logarithmic impedance values obtained before and after hybridization at the different film thickness.

compared to the complementary matched sequences. However, our experiment had only a 14.3% response, as compared to the complementary matched sequence, which was observed for end two-base and center one-base mismatched sequences.

The sensitivity for hybridization was tested as a function of the thickness of the polymer film. Figure 2 shows the difference of logarithmic impedance values obtained before and after hybridization using the probe ODN attached on the polymer films. Adjusting the number of potential cyclings 5 to 10 times controlled thickness of the polymer film. The sensitivity in the difference of logarithmic impedance values using the probe ODN attached on a thinner polymer film (5 times potential cycling) was better than that using a thicker one (10 times) at the frequency range from

100 Hz to 10 kHz. This means that the number of potential cyclings affects the amount of the active probe ODN moiety attached on the surface of the polymer film. The number of the active probe ODN should be proportional to that of the carboxy groups that are attached on the polymer film's surface. There are many more carboxy groups buried inside the thicker polymer film, as compared to the thinner film. In the thicker film, larger amounts of the probe ODN are hindered from orienting toward the outside of the film surface. Thus, the sensitivity of the probe ODN attached on a thinner polymer film was higher than that of a thicker one.

In conclusion, the difference in impedance values before and after hybridization might arise from a change in the conductivity and capacitive current. When compared with other hybridization detection methods reported to date, the presented method has many advantages, such as reducing reaction time without using any indicators or fluorescent materials and higher selectivity for complementary and mismatched target sequences. In addition, the biggest response at a fixed frequency provides for short measuring times using a simple impedance spectrometer. It will bring about the fabrication of a microarray by the electrochemical detection method for the simple diagnosis of diseases.

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