Abstract

Using the redox-active DNA conjugate (ferrocene-modified oligonucleotide 12 mer) as a probe, the novel electrochemical gene sensor, which is sensitive, convenient, and not relying on radio isotope, has been developed. Oligonucleotide (16 mer) complementary to the target (19 mer) was immobilized onto gold electrode through the specific chemisorption of successive phosphorothioates which were introduced into 5'-end of the oligonucleotide. The sequence of the conjugate was designed to be also complementary to another site of the target. Therefore, the conjugate and the oligonucleotide anchoring on the electrode formed a sandwich-type ternary complex with a target DNA to give electric currents based on the ferrocene oxidation. By using this system, we have distinguished the mutant that has one base substitution from the fully complementary target. © 2002 Published by Elsevier Science B.V.

1. Introduction

Bioconjugation between two or more molecules is a smart way for producing the novel molecules possessing combined and synergistic properties. Many natural molecules such as nucleic acids and proteins have been subjected to conjugation with various other natural and synthetic molecules according to the demand [1]. One of the most successful work on proteins should be that for antibody–enzyme conjugates, which are, now, commonly used in non-radioactive ELISA (enzyme-linked immunosorbent assay) techniques in immunoassay. The conjugation of nucleic acids, DNAs and RNAs, with certain chemical groups has also studied and provided the unique nucleic acid conjugates having carefully engineered characteristics [2]. By the conjugation, nucleic acids obtained novel functions such as those for DNA recognition, DNA cleaving activity, fluorescent property for DNA probing, and so on. Here, we have provided redox activity for oligodeoxynucleotide (ODN) to make electrochemically active DNA conjugate.

Electrochemical techniques are potentially sensitive and versatile. The redox reaction of DNA is, however, irreversible and occurs at highly negative and positive potentials in which sensitive detection of the feeble current is difficult because of large solvent decomposition background cur-
rent. Therefore, such conjugate should be useful as an electrochemically active DNA probe that undergoes reversible electrode reactions at less-extreme potentials [3,4]. We have already proposed the methods for electrochemical detection of DNA by using ferrocene-modified ODN as a probe, in which femto mole detection of the target was attainable on HPLC equipped with electrochemical detector [5,6]. Recently, electrochemical study on DNA has been drawing much attention. Several research groups are engaged in the development of novel electrochemically active DNA ligands and their application to DNA detection [7–10], and the cleverly designed methodology for detecting the DNA itself [11–18].

In this paper, we have showed the possibility of electrochemical gene sensor using a model system. ODN-modified Au electrode and ferrocene-ODN conjugate were used as the sensing interface and the probe, respectively [19]. Schematic illustration of the principle of the gene sensor is shown in Fig. 1. Single-stranded ODN, which is complementary to the target, is immobilized onto the gold electrode. This electrode was used as a sensing interface. The sequence of the conjugate used here is also complementary to another site of the target. Therefore, sandwich-type ternary complex should form on the electrode surface when the target is present. The amount of the ferrocene units concentrated onto the sensing interface should be proportional to that of the target in the sample solution. On the other hand, if the added DNA is not the target or has a point mutation, such complex does not effectively form on the interface. Thus, one should be able to differentiate the target from the mutant by monitoring the magnitude of the current based on the redox reaction of the conjugate.

2. Experimental

2.1. Materials

All dimethoxytrityl nucleoside phosphoramidites, Beaucage’s reagent, and support resins for automated DNA synthesis were purchased from Beckman Co. All other reagents used in this study were of the highest grade commercially available and used without further purification. Electrodes used for electrochemical study were purchased from BAS Co. An AT-cut 9 MHz quartz-crystal microbalance (QCM) is commercially available from SEIKO EG&G.

2.2. Preparation of ODNs

ODNs used in this study were prepared on a fully automated DNA synthesizer (Beckman, Oligo 1000). Sequences of the synthesized ODNs are indicated below. While the sequence of p is complementary to f and s, m has a C–C mismatch in the region complementary to s.

\[
\begin{align*}
\text{f (12 mer):} & \quad \text{F} \quad \text{F} \quad \text{F} \quad \text{F} \\
\text{s (16 mer):} & \quad 5' \text{TtTtTtTtTtTtTcTATACATG} 3' \\
\text{p (19 mer):} & \quad 5' \text{CATGTATAAAAAA3' } \\
\text{m (19 mer):} & \quad 5' \text{CATCTTATAAAAAAA3'}
\end{align*}
\]

“s” stands for phosphorothioester bond.
A standard dimethoxytrityl nucleoside phosphoramidite coupling method was used on a 1000 nmol CPG support column. For the synthesis of $s$, Beaucage’s reagent was used to sulfurate successive five phosphate bonds from 5’-end of the ODN; sulfurating was accomplished by the reagent on the oxidation steps in usual ODN synthesis [20]. Liberation from the resin and the following purification and deprotection were carried out according to the procedure described previously [5]. All isolated ODNs were confirmed for their homogeneity by reversed phase HPLC analysis and were stored at $-20\,^\circ\mathrm{C}$ after evaporation. The concentration of single-stranded ODNs was calculated using molar extinction coefficients at 260 nm derived from a nearest-neighbor model [21].

2.3. Synthesis of the redox-active DNA probe ($f$)

The synthesis and purification were carried out according to the method previously reported [5,6]. The product was characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager, PerSeptive Biosystems) and retention time of reversed phase HPLC.

2.4. Preparation of the sensing interface

A well polished Au electrode (1.6 mm φ disk) was used as the base of the sensing interface. Ten microliters of 50 μM aqueous solution (containing 100 mM KCl) of $s$ was placed onto the electrode. After evaporation, the electrode was washed thoroughly with 100 mM KCl solution. Then, this electrode was used as a sensing interface. The modification of the ODN, $s$, onto the electrode was confirmed by cyclic voltammetric measurements (CVs) by using BAS CV-50 W voltammetry analyzer. CVs with the electrode were carried out at 25 °C before and after the procedure of ODN modification in the presence of 100 mM KCl as electrolyte and 10 mM ferrocyanide/ferricyanide redox couple as the marker ions [22,23]. The sensing interface, a Pt plate, and a standard Ag/AgCl (saturated KCl) electrode were used as working, counter, and reference electrode, respectively.

2.5. Infrared spectral (IR) study on ODN modification onto the Au surface

IR measurement was performed on a JASCO FT–IR 620 spectrometer. Reflection–absorption (RA) spectrum was acquired with 80° angles of incidence using a polarizer to eliminate s-polarized light. A gold mirror electrode was placed in the electrochemical cell in contact with an aqueous 100 mM H$_2$SO$_4$ solution containing 10 mM KCl. The electrode was first subjected to anodic potential cycling to give a fresh surface exposed. Fifty microliters of 50 μM aqueous solution of $s$ was placed onto the gold substrate. After evaporation, the substrate was thoroughly rinsed with deionized water, dried under vacuum and then used for measurement immediately.

2.6. Electrochemistry

2.6.1. Differential pulse voltammetry (DPV)

To prepare sample solution, aqueous solutions (containing 100 mM KCl, 5 mM tris–HCl (pH 8.0)) of equimolar mixture, $f$–$p$ and $f$–$m$ (50 μM each), were kept at 90 °C for 10 min. Then, the solutions were allowed to cool gradually for annealing and stored at 5 °C as stock sample solutions [24]. For detecting the target, the sensing interface was immersed in a 30 μl of stock sample solution at 5 °C for 24 h. Then the interface was rinsed with 100 mM KCl solution at 5 °C for washing away the non-specific binding. DPV was carried out using BAS CV-50 W voltammetry analyzer with a conventional design of a three-electrodes system mentioned above (WE: sensing interface, CE: Pt plate, RE: Ag/AgCl (saturated KCl)). The water jacket of the cell was maintained at 5 °C throughout the measurement. The measurements were performed in 100 mM KCl solution under the following conditions: pulse amplitude, 50 mV; pulse width, 50 ms; pulse period, 200 ms; rate, 25 mV s$^{-1}$; temperature, 5 °C.

2.6.2. Quantification of the $f$ on the sensing interface

After the formation of ternary complexes on the sensing interface, the amount of the ferrocene units was estimated from the peak area of CV according
to the procedure previously reported [25]. The measurements were carried out in 100 mM KCl solution using the three-electrodes system as used for DPV measurements under the following conditions: scan rate, 25 mV s$^{-1}$; temperature, 5 °C.

2.7. Quantitative analysis of surface ODN using QCM

QCMs are well known as sensitive mass measuring devices. Their resonance frequency is proved to decrease linearly upon the increase of the mass on the QCM in a nanogram level. The crystal used in this study was supplied as gold coated QCM electrode, which is calibrated to change its frequency by 1 Hz responding to the mass change of 0.98 ng. The diameter of the gold coating moiety was 5.0 mm. The surface of the QCM was cleaned by Cr$_2$O$_7$/H$_2$SO$_4$ (1:1) for a minute, followed by rinsing with pure water and drying with nitrogen.

Thirty microliters of 50 μM solution containing 100 mM KCl was loaded onto the gold surface. After evaporation, the QCM was washed thoroughly with 100 mM KCl. The hybridization experiment was performed by exposing the QCM to aqueous solution of f-p (50 μM) for 4 h at 5 °C. Frequency of the QCM was measured after each procedure (wash, modification, and hybridization). The amount of the ODN immobilized onto the gold surface on each step (modification and hybridization) was estimated from the difference in the frequency between the measurements.

3. Results

3.1. Synthesis of the conjugate (f)

At the final step of the synthesis, the coupling reaction of the terminal amine introduced on 5'-end of the ODN (dT$_{12}$) and a succinimidyl ester of ferrocene carboxylate was carried out in 100 mM NaHCO$_3$–Na$_2$CO$_3$ buffer (pH 9.5) containing 25% dimethylsulfoxide: the solvent composition was optimized considering the solubility of both the reactants (the activated ester still remained undissolved) and the effect of reactivity depression of the activated ester in aqueous media. The HPLC chromatograms of the starting oligonucleotide amine and the reaction mixture are shown in Fig. 2(a) and (b), respectively. Emerged hydrophobic fraction was collected as the desired compound. MALDI-TOF mass spectrometry clearly indicated that this newly generated component was the aimed compound f (found: 3980.50, calc.: 3979.42). The yield for this reaction was relatively high, ca. 70%, though it was carried out under heterogeneous conditions.

3.2. Electrode modification

In this study, phosphorothioate bonds were used as anchors toward the Au electrode. Although, the thiol group can be introduced onto
the terminus of ODN using amidite reagent that has thiol, this reagent is very expensive. On the other hand, phosphorothioate bonds are easily introduced into the synthetic DNA by use of Beaucage’s reagent in the oxidation step of ODN synthesis [20]. Successive five phosphorothioates have been introduced, because the affinity of a phosphorothioate group to Au may be weaker than that of a thiol [26,27].

Cyclic voltammograms of ferrocyanide/ferri-cyanide redox couple with the ODN-modified electrode (sensing interface) and unmodified one are indicated in Fig. 3. While the electrode treated by the ODN, d(TTTTTTCTCATACATG), which does not have any sulfur atoms gave the same voltammogram as the bare, the peak current obtained by s-modified electrode was significantly suppressed. This indicated that the redox couple was excluded from the electrode surface due to the electrostatic repulsion between the anionic redox couple and the ODN polyanion (s) anchoring on the electrode.

The proposed immobilization chemistry was characterized by IR spectroscopy. Fig. 4 shows the RA spectrum of the sulfurated ODN, s, immobilized on a gold substrate. In the RA spectrum, two peaks at 1233 and 1174 cm\(^{-1}\) can be identified to P-O stretches for the antisymmetrical and symmetrical modes, respectively, of the phosphate group of the ODN. In addition, absorption due to the C-O-C stretch of the deoxyribose in the backbone is observed at around 1090 cm\(^{-1}\). The peaks at around 1550 and 1700 m\(^{-1}\), on the other hand, might be caused by the skeletal and base, respectively, in ODN strand.

Table 1 indicates the results of quantitative study using the QCM. The treatment of the QCM with s decreased the frequency of the crystal by 810 Hz, which corresponds to the increase in the weight of 794 ng. The s density on the gold surface is estimated to be 828 pmol cm\(^{-2}\). This highly dense packing of negative charges might give a good account of the significant current suppression observed in CV study mentioned above. Although, this density surpass the theoretical one considering the occupied area by a single-stranded ODN and the apparent area of gold coat on the QCM, the roughness of the gold surface might account for it.

The heat-treatment of the s-modified electrode at 80 °C for 30 min in water did not make any difference in the voltammogram. It is likely that the multi-pod anchoring by phosphorothioates should endorse a stable immobilization of DNA on Au electrode. This newly developed method would be widely useful for fixation of synthetic DNA onto electrode.

### 3.3. Mismatch detection

Fig. 5 shows the differential pulse voltammograms for p and m. A significant anodic peak due to the oxidation of ferrocene moiety was observed for p, perfectly matched 19 mer. It
seemed that the expected ternary complex (Fig. 1) formed on the electrode surface. On the other hand, only a slight peak was observed for a mismatch 19 mer. By using our gene sensing system, one can easily know only one base substitution among 19 bases as a significant difference in magnitude of anodic current.

In the previous study, the redox current of the \( f \) in homogeneous solution was observed at about 400 mV vs. Ag/AgCl reference electrode [5]. On the other hand, the redox potential observed on this system was about 600 mV. The potential was shifted to positive side for as much as about 200 mV. Similar behavior was reported by Letsinger and coworkers for the electrode modified with ferrocene-mononucleotide conjugate [28]. Oxidation of ferrocene to ferrocenium cation requires the concomitant invasion of the counter anion into the ODN layer. This invasion seems to be difficult due to highly dense anionic charges on the surface of the sensing interface. This effect should destabilize the oxidized state, so that the redox potential is appreciably increased.

Slight current observed in the mismatch sample may be diminished by the optimization of the measurement conditions such as immersing period, temperature, salt concentration and/or coexisting non-specific binding blocker. The optimized conditions should promise higher sensitivity. In fact, we have carried out a preliminary study for detection limit of the gene sensor. In this experiment, we succeeded the detection of \( 10^{-15} \) moles of the target. This detection sensitivity seems to be sufficient for practical uses.

3.4. Characterization of the sensing interface

Fig. 6 shows a cyclic voltammogram of the sensor which was treated with \( f - p \). From the peak area of this voltammogram, the density of ferrocene units concentrated on the electrode surface was estimated according to the method previously reported [25]. It was revealed that
Table 1
Quantitative study concerning the modification and the hybridization of the ODN on the gold surface by using QCM

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Difference in</th>
<th>Density (pmol cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (Hz)</td>
<td>Mass (ng)</td>
</tr>
<tr>
<td>Modification of s</td>
<td>−810</td>
<td>794</td>
</tr>
<tr>
<td>Hybridization with f-p</td>
<td>−150</td>
<td>147</td>
</tr>
</tbody>
</table>

23–55 pmol of ferrocene exist per 1 cm² of the electrode surface.

The QCM study would provide the complementary information to that from CV study concerning the hybridization taking place on the sensing interface. The treatment of the s-modified QCM electrode with f–p (hybridization) caused the decrease in the frequency of 150 Hz, which corresponds to the increase in the weight of 147 ng (Table 1). From this weight, the density of the hybridized f–p on the gold surface has been estimated to be 77 pmol cm⁻². This value is comparable with that estimated from the CV study. Comparing the density with that of s, efficiency of hybridization is less than 10%. Recently, Patolsky et al. also reported similar hybridization efficiency on gold surface [18]. This might be, however, reasonable if one consider that the amount of s that is potent for hybridization should be limited because of the intricate electrode surface and the stiffness of double-stranded ODN (f–p).

These results for coverage of the duplex (f–p) were almost the same as those reported by Kelley and Barton [29]. From the density, one can estimate the area occupied by one ferrocene unit to be the circle whose diameter is 15–30 Å. Since the diameter of DNA helix is about 20 Å, the ternary complex, f–p–s, seems densely packed. This provides the extreme anionic charge density on the electrode surface to give unusual shift of the redox potential observed here. However, one should take the roughness of electrode surface into consideration again: real density might be somewhat lower.

Temperature effect on the anodic current of the voltammogram was studied. The results are shown in Fig. 7. The peak for the target sample did not change at all by rinsing the electrode with aqueous KCl solution at 5 and 10 °C. However, the similar treatment at 20 °C diminished the peak current appreciably. On the other hand, the slight current observed for m scarcely decreased by the same treatment (data not shown). These results indicate that the binding of the target was temperature-sensitive. Such property is the peculiar feature of DNA hybridization. That is, the observed interaction taking place on the electrode should be precise molecular recognition based on the Watson–Crick base-pairing as expected.

For practical gene diagnosis, durability and reproducibility as the sensor are one of the important properties. Fig. 8 shows the possibility for repeated use of the sensing interface. Anodic response observed for the target was diminished

Fig. 5. Differential pulse voltammograms of the interface treated by f–p (target) (—) and that treated by f–m (mutant) (---). The ODN electrode was sensitive only for the target, p. The measurements were carried out in 100 mM KCl solution at 5 °C. CE, Pt plate; RE, Ag/AgCl; pulse period, 200 ms; scan rate, 25 mV s⁻¹; pulse amplitude, 50 mV; pulse width, 50 ms.
Fig. 6. Cyclic voltammogram of the interface treated by f-p (target). The measurements were carried out in 100 mM KCl solution at 5 °C. Electrode surface coverage was calculated from the peak area. CE, Pt plate; RE, Ag/AgCl; scan rate, 25 mV s⁻¹.

Fig. 8. The study for repeated use as the gene sensor. The sensing interface which was treated by f-p at 5 °C (a) was washed with 100 mM KCl solution at 25 °C (b) and re-hybridized with f-p at 5 °C (c); DPV measurements were carried out at each step. CE, Pt plate; RE, Ag/AgCl; pulse period, 200 ms; scan rate, 25 mV s⁻¹; pulse amplitude, 50 mV; pulse width, 50 ms.

Fig. 7. Temperature dependence of the signals. Peak currents obtained by the DPVs of the interface treated with f-p (target) were plotted against the measurement temperatures. All measurements were carried out 10 min after reaching the temperatures under the same conditions of Fig. 5.

appreciably by washing with aqueous KCl at 25 °C. And re-hybridization at 5 °C has completely restored the anodic peak of the voltammogram. These behaviors clearly indicated the potential for repeated use of the interface as a practical sensor.

Comparing the Figs. 5 and 8, the discrepancy is apparent in the magnitude of the response between the interfaces. This should be due to the difference in the gold surface conditions of the electrodes used as the basis of sensing interfaces. Subtle irregularity in the procedure for immobilizing ODN may also account for it partly. This inequality in the performance between the interfaces remains to be dissolved.
4. Discussion

So far several electrochemical gene sensors using electrochemically active DNA ligands have been proposed by some other groups [10,30–33]. The principle of these sensors is common, being summarized as follows. Single-stranded ODN which is complementary to the target is immobilized onto the electrode. If the single-stranded target is added into the solution where the electrode is immersed, double-stranded complex forms on the electrode surface. Then, coexisting redox-active probes such as daunomycin and cobalt–polypiridine complex are concentrated onto the double-stranded DNA on the electrode surface. The electric current due to the probes should be relevant to the amount of the target. Although this method has generality, the detection limit should depend on the probe's preference for double-stranded DNA against single-stranded one. However, in general, the selectivity of the probes is not so high. Binding of the probe to immobilized single-stranded ODN on the electrode causes background current, resulting in the limitation of the sensitive response to the target. That is, the probes have intrinsic and unavoidable limit depending on the preference.

On the other hand, our strategy described here is totally different from conventional gene sensors. The electrochemical response in our sensor is based on the hybridization between ODNs, which is highly specific and used in the most of usual method for gene analysis. Therefore, the techniques of DNA hybridization have been attained full growth; many tips and know-how are available for improving the sensitivity. These assets from the studies of probe hybridization on solid surface should help us to brush up the system and apply this sensor to practical various samples containing many contaminants. For example, the sensitivity depends on the balance of the responses between for fullmatch- and mismatch samples. Although longer sequences would give strong signals, they have much tolerance for mispairing. The reverse is true for shorter sequences. The conditions optimized for given complementary length of the samples could be determined by taking advantage of the accumulated knowledge about the hybridization in heterogeneous systems. Therefore, while we only used seven bases complementary length here, this system should be easily applied to longer complementary length.

When applying the sensor to gene diagnosis, we have to consider some points relating with practical demands. First point is the versatility of our sensor toward the target. There is a possibility that the geometry or direction of the probe in the ternary complex may change the distance between the ferrocene unit and the electrode surface. The distance should affect the response in electric current. Besides one may worry that the length of the target would change the distance; longer target makes the distance longer. However, the sequence targeted by the gene sensor is usually that of already-known at least for the wild type. Therefore, one can design the sequences of the probe (including the direction of ferrocene unit) and the immobilized ODN to be complementary to the adjacent sites in the target in order to bring the ferrocene unit near the electrode surface. In addition, we have shown in the previous study that the redox properties of the probe scarcely depend on the neighboring extra sequence, with which the ferrocene moiety of the probe could be in contact [5]. Therefore, we can tune the sensing system whose response does not depend on the length and the sequence of the target. Notwithstanding, steric effect of the much longer target might remain even in our system. The steric hindrance may prevent the concentration of the target to some extent. As a result, the amount of the ferrocene units on the electrode should be decreased. Pretreatment of the sample by a certain exonuclease would suppress such an effect.

Another point is the time required for the measurement. Now, we are in preliminary stage of studying the gene sensor. The measurement conditions described here are not optimized yet. We immersed the sensing interface in the sample solution for 24 h to make sure, although such a long period should not be required for attaining the equilibrium because the formation of double-stranded complex of DNA is quick. We can, therefore, make the measurement time shorter and shorter according to the demands on practical use.
5. Conclusions

Electrochemical gene sensor has been developed using our ferrocene-ODN conjugate, f, and the ODN anchoring on a gold electrode, s. The sensor allowed us to discriminate the point mutation under the moderate oxidation potential, in which the measurements were not hampered by solvent decomposition background current at all. We believe that the present gene sensing method, which is convenient and provide high detection sensitivity, is applicable to practical gene diagnosis.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Encouragement of Young Scientists (No. 08750941) and by a Grant-in-Aid for Scientific Research (B) (No. 09555266) from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT). The authors are also grateful to The Sagawa Foundation for Promotion of Frontier Science (to T.I.) and to Terumo Life Science Foundation (to M.M.).

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