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Label-free Ratiometric Electrochemical DNA Sensing Based on β-Cyclodextrin-modified Probe Immobilized on Ferrocene Monolayers

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We have developed a novel label-free oligonucleotide sensor that possesses β-cyclodextrin (β-CD) as a hybridization-amenable signal repressor and ferrocene (Fc) as a signal generator with two different redox potentials for single-stranded and double-stranded states. The probe gives us an electrochemical ratiometric response based on the redox currents at these two potentials, revealing an oligonucleotide recognition event. The sensor is based on an artificial oligonucleotide probe, β-CD-DNA-SH, and FcUT immobilized on gold electrode surfaces. Electrochemical measurements showed two redox peaks of Fc redox reactions, with the higher potential peak corresponding to the redox reaction of Fc forming the inclusion complex with the terminal β-CD, and the lower one corresponding to the redox reaction of Fc not forming the inclusion complex. The ratios of these two peak currents were 0.815 and 0.398 for the target DNA and mismatched DNA, respectively. A sequence-specific electrochemical ratiometric response was observed. The sensors prepared from the developed probe are anticipated to detect oligonucleotides even when a deterioration in effectiveness might be expected due to rough treatment such as repeated use or unsuitable storage conditions. To the best of our knowledge, this is the first report of a ratiometric electrochemical oligonucleotide sensor based on one signal-generating moiety with two redox potentials.

1. Introduction

Nucleotides play important roles as carriers of genetic information. In living things, DNA stores genetic information, and items of genetic information are released as RNA.[1] RNA is released in response to stimuli to which living things are exposed.[2] If we can effectively exploit these oligonucleotides as biomarkers, we can obtain information on the state of these living things in addition to genetic information.

Many researchers are currently focusing on the development of novel oligonucleotide detection techniques. For instance, detection techniques for fish DNA released into the

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environment (environmental DNA, eDNA) are increasingly being studied as novel alternatives to conventional ecological survey techniques.[3–6] MicroRNAs (miRNAs) found in blood samples from patients are of interest as biomarkers for the early detection of cancer cells.[7–10] Moreover, RNAs extracted from model cells exposed to chemicals are utilized to develop new methods of evaluating chemical toxicity as alternatives to conventional animal tests.[11–14]

Most of these oligonucleotide detection techniques require benchtop apparatus in laboratories, including next-generation sequencers and DNA microarrays. However, the rising demand for in situ detection in the environmental and biomedical fields is creating a growing need for rapid and simple oligonucleotide detection techniques.

In response to this demand, we have studied oligonucleotide sensors without labeling the target oligonucleotides, based on electrochemical methods that are advantageous in developing space- and energy-saving onsite techniques.[15–17] We have particularly focused on artificial oligonucleotide probes that possess signal-generating moieties whose signals fluctuate upon molecular recognition (hybridization) by target oligonucleotides. These are probes with a switch function that converts molecular recognition events into signal changes. These sensors, which incorporate probes immobilized on the electrode surfaces, make it possible to detect oligonucleotides by simply immersing the sensors in oligonucleotide sample solutions without adding any other reagents.

We have synthesized an oligonucleotide probe based on a mechanism in which the electrochemical signal from the terminal ferrocene (Fc) changes from ON to OFF ('signal-off' architecture) upon hybridization.[18,19] Sensor arrays based on these probes were fabricated to demonstrate the detection of oligonucleotide biomarkers.[20] We have also developed a probe with Fc at one terminal and β-cyclodextrin (β-CD) at the other, based on a mechanism by which the electrochemical signal from the Fc changes from OFF to ON ('signal-on' architecture) on hybridization.[21] The probe has an inclusion complex between Fc and β-CD without the target, and the complex is dissociated by the formation of a rigid double-stranded structure when the probe forms a hybrid with the target, resulting in the restoration of the redox activity of the Fc.

In the sensors on these probes, hybridization of one probe with one target results in a signal change from one Fc. As a result, the observed redox current is dependent on the number of effective probes (i.e., the number of active Fcs) on the sensor surfaces. Reproductive sensor preparation can be controlled according to the number of probes on the sensor surfaces, but multiple use of the sensors and unsuitable storage conditions may cause the deterioration of sensor surfaces and changes in the number of effective probes from the initial state (see Ref. 18 for an example). In this case, it is difficult to tell whether the observed current change is a result of hybridization or due to a change in the number of active probes.

Therefore, toward solving this problem, we used a signal-generating moiety with two different redox potentials for single-stranded and double-stranded states. The probe gives a ratiometric electrochemical response based on the redox currents at these two potentials to indicate an oligonucleotide recognition event. To the best of our knowledge, this is the first report of ratiometric electrochemical oligonucleotide sensors based on one signal-generating moiety with two redox potentials.[22–24] We synthesized a β-CD-terminated artificial probe and prepared sensors by immobilizing the probe on a gold electrode surface with a short-chained

thiol. We previously demonstrated that the association of an inclusion complex between Fc and β-CD positively shifts the redox potential of Fc.[21] In a single-stranded state, the redox potential of Fc is the higher one because the terminal β-CD forms an inclusion complex with Fc on the sensor surface. In a double-stranded state, however, the redox potential of Fc is the lower one because the inclusion complex is dissociated due to the formation of the rigid probe/target structure.

In this paper, we report the synthesis of the probe, the preparation of the sensors, and a demonstration of label-free DNA detection. The artificial oligonucleotide probe, β-CD-DNA-SH, was prepared from the custom synthesis of a precursor DNA having a bicyclo[6.1.0]nonyne (BCN) moiety and its copper-free click-chemistry reaction with β-CD-N_3.[25,26] The sensor was prepared by immobilizing β-CD-DNA-SH and FcUT on the surfaces of polished gold electrodes. The working principle is depicted in Fig. 1. Cyclic voltammograms (CVs) and square wave voltammograms (SWVs) were recorded for the prepared sensor in the electrolyte solutions, showing two redox peaks of Fc redox reactions on the sensor surfaces. We believe that the higher potential peak is due to the redox reaction of Fc forming an inclusion complex with the terminal β-CD of the β-CD-DNA-SH and that the lower potential peak is due to the redox reaction of Fc not forming the inclusion complex. The SWV for the prepared sensor soaked in a solution containing a complementary target oligonucleotide showed a relative increase in the redox current at the lower potential peak. This is because the hybridization-triggered formation of a double-stranded structure made the probe structure rigid, resulting in the dissociation of the inclusion complex of β-CD with Fc on the sensor surface. On the other hand, the SWV for the prepared sensor soaked in a solution containing urea showed a relative increase in the redox current at the higher potential peak. This is because urea causes denaturation of the double-stranded structure by inhibiting hydrogen bonding, and a flexible single-stranded probe

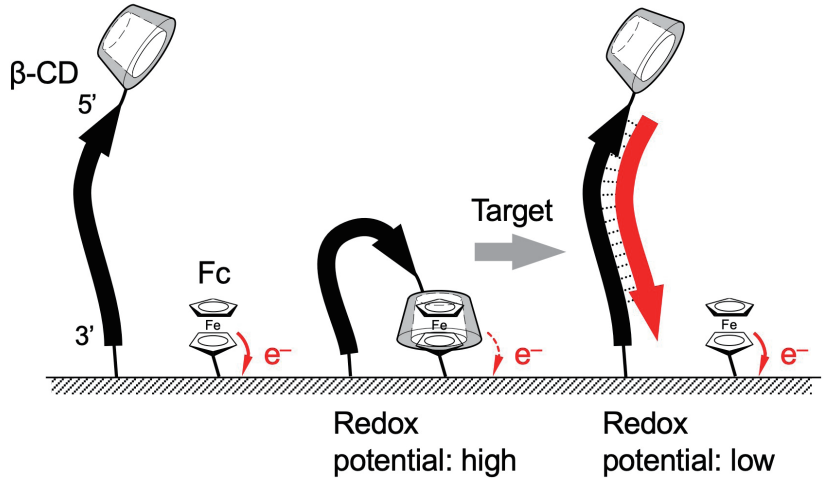

Fig. 1. (Color online) Working principle of the probe β-CD-DNA-SH detecting the target oligonucleotide based on the hybridization-triggered dissociation of an inclusion complex of β-CD with Fcs on the sensor surface. The redox potential of Fcs forming an inclusion complex with β-CD is higher than that of uncovered Fcs, enabling ratiometric electrochemical detection.

structure allows the formation of an inclusion complex of β-CD with Fc on the sensor surface. The results of this study indicate the potential for oligonucleotide sensing by utilizing the redox peaks of the Fc redox reaction on an electrode surface.

2. Materials and Methods

2.1 Reagents and apparatus

The artificial oligonucleotide probe, β-CD-DNA-SH (**3** in Fig. 2), was prepared from the custom synthesis of a precursor DNA and its click reaction with β-CD-N_3 (**2** in Fig. 2). The precursor DNA, BCN-DNA-SH (**1** in Fig. 2), was purchased from Nihon Gene Research Laboratories (Sendai, Japan). It was designed as a conjugate of DNA and other moieties, with the structure of 5' BCN-C3-GCA ACC TTC CCT ATT ACT CCA C-C3-$(CH_2)_6$-S-S-$(CH_2)_6$OH 3' with the sequence from the estrogen-responsive gene EGR3 [accession number: X63741; National Center for Biotechnology Information (NCBI)[20,27]], where BCN and C3 denote a BCN moiety and a linker with a carbon length of 3, respectively. The thiol moiety was protected by a disulfide bond in the β-CD-DNA-SH probe, but the probe was used in this study without deprotection. 6A-azido-6A-deoxy-β-cyclodextrin (β-CD-N_3) was from Tokyo Chemical Industry (Tokyo, Japan). The structures of the BCN-DNA-SH, β-CD-DNA-SH, and β-CD-N_3 are depicted in Fig. 2. The DNA was from Eurofines Genomics Japan (Tokyo, Japan), the sequences of which were 5' GTG GAG TAA TAG GGA AGG TTG C 3' (target DNA),

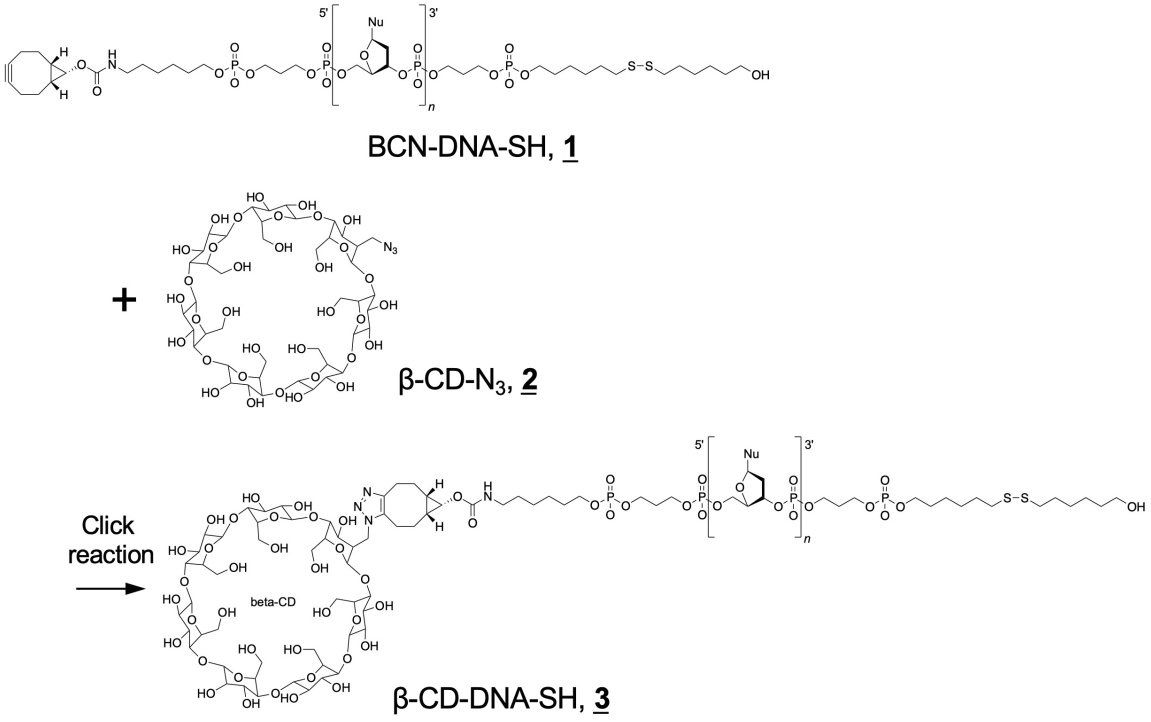

Fig. 2. Synthetic scheme for the probe β-CD-DNA-SH.

complementary to the sequence of the probe, and 5' TAG CTT ATC AGA CTG ATG TTG A 3' (mismatched DNA). 11-Ferrocenyl-1-undecanethiol (FcUT) was from Dojindo (Kumamoto, Japan). KOH, H_2SO_4, 10X phosphate-buffered saline (10X PBS), $NaClO_4$, and urea were from Fujifilm-Wako Chemical (Tokyo, Japan). 1X PBS was prepared from 10X PBS by tenfold dilution. All other chemicals used were of analytical reagent grade. All aqueous solutions were prepared with deionized and charcoal-treated water (specified resistance > 18.2 MΩ cm) obtained using a Milli-Q reagent-grade water system (Merck-Millipore; Bedford, MA).

2.2 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectra were measured on an AXIMA-CFR plus mass spectrometer (Shimadzu; Kyoto, Japan) in linear mode with positive polarity using 2,4,6-trihydroxy-acetophenone as the matrix and ammonium acetate as the cationizing agent. A sample aqueous solution, a 10 mg/mL acetonitrile/water (1:1 v/v) solution of the matrix, and a 50 mg/mL aqueous solution of the cationizing agent were mixed together at a ratio of 1:8:1 v/v.

2.3 Synthesis of the probe

The click-chemistry reaction between a BCN moiety and an azide moiety proceeded without using copper as a catalyst, as in classical click chemistry.[25,26] 100 μM BCN-DNA-SH, **1**, and 1 mM β-CD-N_3, **2**, were prepared with 1X PBS. 193 μL of 100 μM BCN-DNA-SH was mixed with 42.8 μL of 1-mM β-CD-N_3 at room temperature overnight. The prepared probe β-CD-DNA-SH (**3**, 90.9 μM in 1X PBS) was identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: β-CD-DNA-SH (8671.17 g/mol): m/z = 9015.17 $[M + 16Na]^+$.

2.4 Preparation of oligonucleotide sensors

Gold disk electrodes (area: 2 mm^2) were purchased from Bioanalytical Systems (BAS) (Tokyo, Japan) and used in all the electrochemical experiments. The electrodes were prepared in accordance with previous reports.[18,19,28,29] Briefly, the electrodes were mechanically polished with wet 0.3 μm and 0.05 μm alumina slurries (Buehler; Lake Bluff, IL). The electrodes were electrochemically polished by cycling the potential between −0.4 and −1.2 V in 0.5 M KOH solution until the CV stabilized. The electrodes were then soaked in 1 M H_2SO_4 solution, followed by cycling the potential between 0 and +1.6 V until a constant CV was observed. The prepared gold electrode was soaked in 1 mM FcUT ethanolic solution at room temperature for 2 h. After washing with ethanol, 90.9 μM β-CD-DNA-SH was added dropwise onto the electrode surface and the electrode was stored at room temperature for 20 h. After washing with water, electrochemical measurements were performed.

2.5 Electrochemical measurements

The electrochemical measurements were performed using a three-electrode configuration of the prepared gold electrode as the working electrode, a Ag/AgCl reference electrode (internal solution: 3 M NaCl), and a Pt auxiliary electrode using an ALS 760C electrochemical analyzer and ALS 760C analysis software (BAS). Cyclic voltammetry and square wave voltammetry were conducted in 1X PBS containing 0.1 M $NaClO_4$. For cyclic voltammetry, the potential was scanned from 0 to +0.8 V and again back to 0 V at a scan rate of 0.1 V s^{-1}. For square wave voltammetry, the potential was scanned from 0 to +0.8 V at a potential step of 2 mV, a potential amplitude of 10 mV, and a frequency of 50 Hz. The sensor responses were expressed as the ratio of redox peak currents at the two peak potentials for the Fc redox reaction. Each SWV was subjected to a baseline correction algorithm in the ALS 760C analysis software to subtract the baseline from the recorded SWV.

3. Results and Discussion

First, we electrochemically investigated the prepared sensor possessing β-CD-DNA-SH and FcUT via CVs (Fig. 3) and SWVs (Fig. 4). The recorded CVs showed a few peaks that were attributable to the redox reaction of Fc moieties on the electrode surface. This indicates that the presence of Fc moieties varies under different circumstances. In general, it is known that the redox peaks of redox-active monolayers are composed of an ensemble of a redox peak of each redox-active moiety. A conformation may cause multiple formal potentials to exist in the ensemble of redox centers.[30] From this, a random conformation of Fc moieties on the electrode surfaces gives a broad redox peak, but an Fc monolayer with high conformation yields a sharp

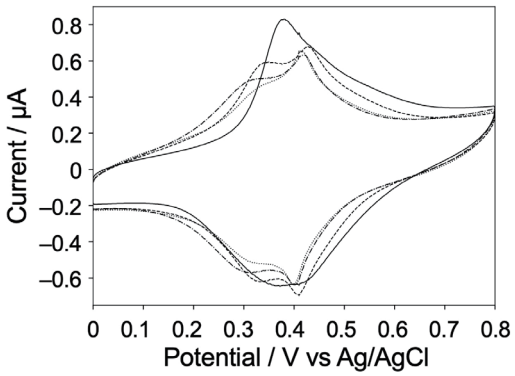

Fig. 3. CVs for the redox reaction of Fc for the FcUT + β-CD-DNA-SH-modified gold disk electrode in 0.1 M NaClO4 + 1X PBS before (solid line) and after incubation in 1 µM target DNA + 1X PBS (dashed line), incubation in 1 µM target mismatched DNA + 1X PBS (dotted line), or incubation in 2 M urea + 1X PBS (dash-dotted line), scanned from 0 to +0.8 and again back to 0 V at a scan rate of 0.1 V s^{-1}.

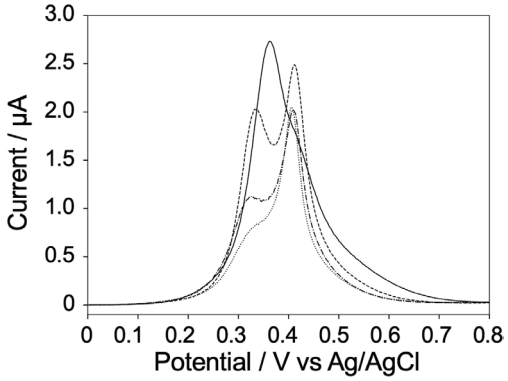

Fig. 4. SWVs for the redox reaction of Fc for the FcUT + β-CD-DNA-SH-modified gold disk electrode in 0.1 M $NaClO_4$ + 1X PBS before (solid line) and after incubation in 1 µM target DNA + 1X PBS (dashed line), incubation in 1 µM target mismatched DNA + 1X PBS (dotted line), or incubation in 2 M urea + 1X PBS (dash-dotted line), scanned from 0 to +0.8 at a potential step of 2 mV, a potential amplitude of 10 mV, and a frequency of 50 Hz.

redox peak. From the SWV obtained under the same conditions, a large redox peak was found at 0.362 V and a small redox peak at 0.408 V. Similar peaks were found in the CV.

We then dipped the sensor into 1 μM target DNA in 1X PBS for 45 min and recorded CVs and SWVs. The CV showed two obvious redox peaks and, more clearly, the SWV presented these two peaks at 0.332 and 0.412 V with currents of 2.03 μA (I_{p_lower}) and 2.49 μA (I_{p_higher}), respectively. This is probably due to the conformational changes of the FcUT monolayer to more stable states induced by applying potentials via electrochemical measurements. The difference between these redox peaks is a function of the resistance to oxidation of the Fc moieties under the two different conditions. The Fc moieties at the higher redox potential are more difficult to oxidize than the Fc moieties at the lower potential.

The formation of an inclusion complex between these two moieties inhibits the redox reaction: their redox potential is clearly shifted to higher potentials.[21,31–33] The Fc moieties for the higher potential probably form inclusion complexes on the electrode surface with β-CD moieties at the terminal of the probe molecules. In contrast, those for the lower potential appear not to form inclusion complexes and are in uncovered forms. The ratio between these two currents at the lower and higher potentials (I_{p_lower}/I_{p_higher}) was calculated to be 0.815.

Next, we dipped the sensor into 2 M urea in 1X PBS for 45 min and recorded CVs and SWVs. The CV showed almost the same response as that for the target DNA, but the SWV showed a more drastic change. The redox current at the lower potential significantly decreased, while the current for the higher potential decreased less, where I_{p_lower} and I_{p_higher} were 1.12 and 2.02 μA, respectively. The ratio between these two currents at the lower and higher potentials (I_{p_lower}/I_{p_higher}) was calculated to be 0.457, a decrease of 43.9% compared with that of the target DNA. This indicates that the number of uncovered Fc moieties decreased and that the number of those forming inclusion complexes with β-CD moieties relatively increased. This is because urea molecules inhibit hydrogen bonding between the probe/target double-stranded structure and the β-CD at the terminal of the flexible single-stranded probe that approaches the Fc on the sensor surface to form an inclusion complex.

Electrochemical measurements for another DNA (mismatched DNA) non-complementary to the probe were also conducted. We dipped the newly prepared sensor into 1 μM mismatched DNA in 1X PBS for 45 min and recorded CVs and SWVs, resulting in similar responses, with I_{p_lower} and I_{p_higher} being 0.816 and 2.05 μA, respectively. The ratio between the two currents at the lower and higher potentials (I_{p_lower}/I_{p_higher}) was calculated to be 0.398, a decrease of 51.1% compared with that when using the target DNA. This indicates that, for the non-complementary DNA, a large number of the Fc moieties form inclusion complexes, similar to the case with urea.

The repeatability of the present method was also confirmed. The values of I_{p_lower}/I_{p_higher} for the target DNA, mismatched DNA, and urea are listed in Table 1.

Table 1
Redox peak currents at lower and higher potentials observed in SWVs measured in the target DNA, mismatched DNA, and urea solution containing 1X PBS. The data for electrochemical measurements repeated twice are listed.

	Target DNA		Mismatched DNA		Urea
I_{p_lower}	2.03	2.04	0.816	0.818	1.112
I_{p_higher}	2.49	2.45	2.05	1.92	2.02
I_{p_lower}/I_{p_higher}	0.815	0.833	0.398	0.426	0.457

4. Conclusions

We have developed a novel label-free oligonucleotide sensor that possesses β-CD as a hybridization-amenable signal repressor and Fc as a signal generator with two different redox potentials for single-stranded and double-stranded states. The probe gives us a ratiometric electrochemical response based on the redox currents at these two potentials, revealing oligonucleotide recognition events. To the best of our knowledge, this is the first report on ratiometric electrochemical oligonucleotide sensors based on one signal-generating moiety with two redox potentials. A sequence-specific response was demonstrated. The difference in the ratiometric electrochemical response between complementary and non-complementary DNA was 51.1%. Repeatability was also confirmed.

Probe molecules with an electrochemical switching function, including the probe developed in this study, are immobilized on the surface of electrodes to convert molecular recognition events to changes in redox currents. However, multiple usage of the sensors and unsuitable storage conditions may cause the deterioration of sensor surfaces and change the number of effective probes from the initial state. If this happens, it is difficult to tell whether the observed change in current is caused by hybridization or a change in the number of probes. The present sensing technique, however, expresses two sensing states, detection or non-detection of oligonucleotides, as two distinct Fc redox potentials. It is therefore expected that the sensor responses will not be affected by the number of Fcs on the sensors. We believe that this working principle will contribute to the increased viability of sensors, in that they are not affected by the deterioration of sensor surfaces.

Acknowledgments

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