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A novel oligonucleotide probe was designed to provide a self-reporting 'signal-on' DNA detection. The probe was terminated with a ferrocene moiety at one end and a β-cyclodextrin moiety at the other end, as the signal-generating and signal-suppressing parts, respectively. Electrochemical measurement of the probe using a carbon-based interdigitated array microelectrode chip showed a sigmoidal current–potential curve in the absence of the target oligonucleotide complementary to the probe. In the presence of the target, however, the curve shifted towards a more negative potential (62 mV) and the observed current at +0.3 V was increased 5.2-fold, indicating that the probe provided a 'signal-on' response upon hybridisation. This probe achieves simple DNA detection without the need to label the target oligonucleotides or to add any other electroactive reagents to the measurement system.

Keywords: DNA detection; signal-on; label-free; ferrocene; β-cyclodextrin

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Introduction

Spectroscopic DNA detection is a commonly employed technique based on fluorescence labelling of the target DNAs (*1*). It is currently a standard technique for DNA detection, but requires time-consuming labelling of the target DNAs. In the search for simpler techniques, a number of electrochemical detection methods without labelling treatments have been developed (*2*). These include types based on electroactive diffusion species (*3–7*), intercalating or groove-binding species (*8–11*), ligation of nucleobases labelled with electroactive species (*12–15*) and methods based on more than one mediator (intercalators, groove binders or nucleobases) and diffusion markers (*16–19*).

The authors, in a previous report, achieved detection of target DNAs using a gold electrode modified with a probe peptide nucleic acid (PNA) possessing a ferrocene (Fc) moiety as the signal part at one end and cysteine as an anchor part at the other end (Fc-PNA) (*20*). Sequence-specific hybridisation induced an increase in the rigidity of the probe structure that inhibited the access of the terminal Fc moiety to the electrode surface and thus decreased the redox current of the Fc moiety. A decrease in the redox current specifically indicated the presence of the target DNA. This method was reported as a simplified detection method that dispensed with the need to label the target DNAs or to add external electroactive species, with a detection limit of 1.4×10^{-11} M. The detection limit was better than those in contemporary reports on similar detection mechanisms using DNA as probes, probably due to the advantages of the PNA's structural flexibility and hybrid stability (*21–23*). Although having a rather lower detection limit, this sensing method utilises the decrease in the redox reaction of the terminal Fc moiety, when the distance between the moiety and the sensor surface is increased: namely, the detection mechanism is a function of the 'signal-off' architecture.

With the aim of achieving more sensitive detection, we studied 'signal-on' type probes emitting electrochemical signals that arise upon hybridisation to enhance hybridisation-amenable changes in probe flexibility. The probe is designed to terminate with an electrochemical signal-generating and signal-suppressing part at opposite ends (Scheme 1). In single-stranded form, the probe is so flexible that the terminal moieties form an intramolecular inclusion complex with each other, suppressing the redox reaction of the signal-generating part. Upon hybridisation, however, the probe structure becomes more rigid and the complex is pulled apart, restoring the original activity of the signal-generating part ('signal-on') (Scheme 2). Although several 'signal-on' detection methods have been reported (*22–25*), their requirements for the use of stem structures or extra oligonucleotides to maintain complicated probe steric structures cause low generality in probe sequences. In contrast, in the studied approach, arbitrary sequences are available without any constraints on the design of the probe sequences. The probe design thus has an advantage over the previous probe designs, in that it allows simple structures to be employed.

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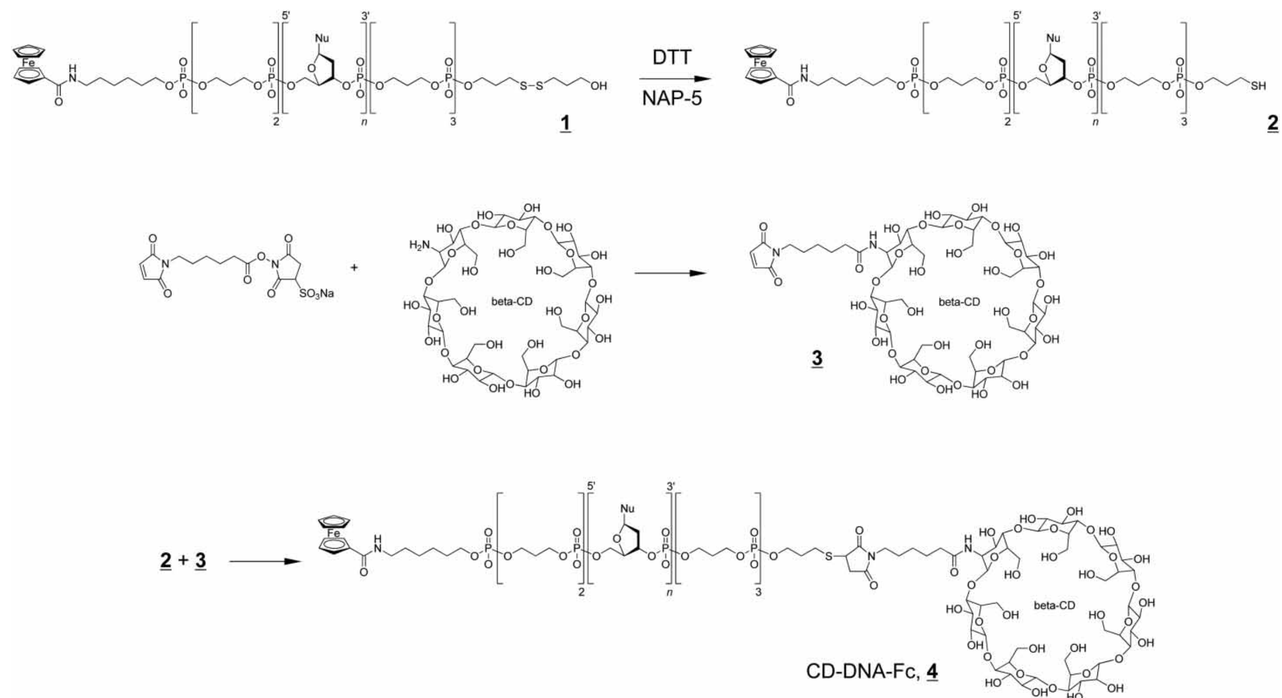

Scheme 1. Synthetic scheme for the probe, CD-DNA-Fc.

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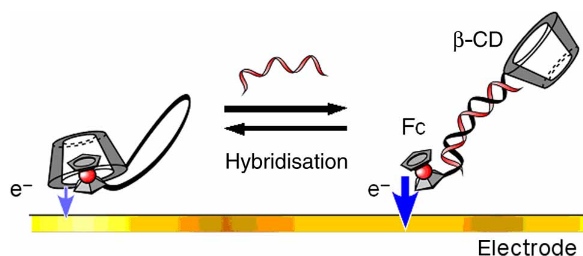
In this study, the probe was designed to terminate with Fc and β-cyclodextrin (β-CD) moieties at both the ends as the signal-generating and signal-suppressing parts, respectively. The synthesised probe, CD-DNA-Fc, showed a sigmoidal current–potential curve in electrochemical measurements, using a carbon-based interdigitated array (IDA) microelectrode chip. In the presence of the target oligonucleotide complementary to the probe, the curve shifted towards a more negative potential (62 mV), and the observed current at +0.3 V increased more than 5-fold (from 1.31 to 6.91 nA), indicating that the probe provides a 'signal-on' response upon hybridisation. This is the first study of a probe realising self-reporting 'signal-on' DNA detection based on the control of the hybridisation-sensitive redox signal by suppression and restoration of its own electrochemical activity.



Scheme 2. Working principle of the synthesised probe, CD-DNA-Fc, detecting the target oligonucleotide based on the intramolecular complex formation/disassociation between the Fc moiety and the β-CD moiety sensitive to hybridisation with the target oligonucleotide complementary to the probe.

Experimental

Reagents and apparatus

A precursor of the probe, an oligonucleotide conjugated with a Fc and a thiol moiety, was purchased from Fasmac (Kanagawa, Japan). Its structure was 5′ Fc-C6-$(C3)_2$-GCA ACC TTC CCT ATT ACT CCA C-$(C3)_3$-$(CH_2)_3$-S-S-$(CH_2)_3$OH 3′, **1**, where Fc, C6 and C3 denote the Fc group, a linker with a carbon length of 6 and a linker with a carbon length of 3, respectively (Scheme 1). The thiol moiety was protected by a disulphide bond in precursor **1**. The length of the probe sequence was selected as 22 bases, following the previous report (*20*). A number of articles reported that short single-stranded DNAs (12–30 bases) tethered on gold surfaces have conformational flexibility, and that, after hybridisation, the consequent double-stranded DNAs acquire more rigid structures (*26–31*). The target oligonucleotide, with the sequence of 5′ GTG GAG TAA TAG GGA AGG TTG C 3′, was purchased from Operon Biotechnologies (Tokyo, Japan). Its extinction coefficient at 260 nm at 25°C was calculated by nearest-neighbour approximation (*32*). The concentrations of the strands were determined using the calculated extinction coefficient of 1.95×10^5 and $2.29 \times 10^5\,M^{-1}\,cm^{-1}$ for the probe and the oligonucleotide strands, respectively.

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One hundred micromolar aqueous solutions of the synthesised probe and the oligonucleotide were prepared and stored as stock solutions at −20°C until use. 3A-amino-3A-deoxy-(2A*S*,3A*S*)-β-cyclodextrin (amino-β-CD) was purchased from Tokyo Chemical Industry (Tokyo, Japan). *N*-(6-maleimidocaproyloxy)sulphosuccinimide sodium salt (sulpho-EMCS) and 6-hydroxymethylferrocene (HMFc) were purchased from Dojindo (Kumamoto, Japan). All other chemicals used were of analytical reagent grade. All aqueous solutions were prepared with deionised and charcoal-treated water (specific resistance > 18.2 MΩ cm) obtained with a Milli-Q reagent grade water system (Millipore, Bedford, MA, USA).

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionisation time-of-flight mass spectra were measured on a Voyager DE PRO mass spectrometer (Applied Biosystems, Tokyo, Japan) in a linear mode with positive polarity using 2,4,6-trihydroxyacetophenone as the matrix and diammonium hydrogen citrate as the cationising 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 cationising agent were mixed together at a ratio of 1:8:1 v/v.

Synthesis of the probe

Fifty microlitres of 1 OD precursor **1** aqueous solution was mixed with 5 μl of a 0.1 M phosphate buffer solution (pH 7.0, Na^+ salt) containing 1 M dithiothreitol, and was placed at 37°C for 1 h to deprotect the disulphide moiety in **1**. To the solution, 150 μl of water was added and the total solution was added to a Sephadex G-25 (NAP-5) gel-filtration column (GE Healthcare, Buckinghamshire, UK) equilibrated in advance with approximately 10 ml of water. Eluted samples were collected for every addition of 0.5 ml of water to the column. The concentration of the deprotected precursor **2** in the collected fraction was measured using a UV-2450 UV–vis spectrometer (Shimadzu, Kyoto, Japan). The fraction with the largest absorbance at 260 nm was used for the next synthesis step. The concentration of the yielded molecule **2** (Scheme 1) was 7.51 μM in the obtained fraction (500 μl). Five hundred microlitres of a 0.1 M phosphate buffer solution containing 5 mM amino-β-CD, 0.1 M NaCl and 1 mM EDTA, and 125 μl of a 0.1 M phosphate buffer containing 0.1 M NaCl and 4 mM sulpho-EMCS were mixed together and stirred at 30°C for 1 h, yielding a β-CD derivative with a maleimide moiety **3**. Addition of 9.39 μl of this solution to the prepared total solution of molecule **2** was followed by standing the solution at 4°C for 20 h. The raw product was purified on an NAP-5 column, yielding molecule **4** as the probe, CD-DNA-Fc (Scheme 1). CD-DNA-Fc (9113.05 g/mol): $m/z = 9100.30$ $[M + H]^+$. After concentrating the CD-DNA-Fc solution by evaporating water from the solution, 5 μl aliquots of 100 μM of a CD-DNA aqueous solution were prepared and stored at −20°C until use.

Electrochemical measurements

A carbon-based IDA (width, 10 μm; gap, 5 μm; length, 2 mm; number, 65) microelectrode chip was purchased from Bioanalytical Systems (BAS, Tokyo, Japan). An Ag/AgCl reference electrode was formed on the chip by coating a corresponding carbon electrode with Ag/AgCl paste (BAS) and then by heating the chip at 120°C for 5 min. Cyclic voltammetry was performed at 25°C using an ALS-730C electrochemical analyser (BAS) with the four-electrode configuration consisting of dual-working interdigitated electrodes, the prepared Ag/AgCl reference electrode and an auxiliary electrode, which were patterned on the chip. The potential at one working electrode was set at 0 V, and the other was scanned from 0 to +0.6 V and again back to 0 V at a scan rate of $0.01\ V\,s^{-1}$. In electrochemical measurements for CD-DNA-Fc in the absence of the complementary target oligonucleotide, a 0.01 M NaCl + 0.1 M phosphate buffer solution (pH 7.0, Na^+ salt) and a CD-DNA-Fc stock solution were mixed together at a ratio of 3:1 v/v. The final concentration of CD-DNA-Fc was 25 μM in the measurement solution. In the electrochemical measurements for CD-DNA-Fc in the presence of the oligonucleotides, a 0.02 M NaCl + 0.2 M phosphate buffer solution, a CD-DNA-Fc stock solution, a target oligonucleotide stock solution and water were mixed together at a ratio of 3:2:2:1 v/v. The final concentrations of both CD-DNA-Fc and the target oligonucleotide were 25 μM in the measurement solution. A 5 μl aliquot of these mixed solutions was dropped on the IDA, the drop was covered with a glass plate (size, 4 × 6 mm; thickness, ca. 150 μm; Matsunami Glass, Osaka, Japan) to prevent the drop from drying out and cyclic voltammograms (CVs) were recorded. A CV measured in the buffer solution without CD-DNA-Fc or any oligonucleotides was used as a baseline subtracted from the observed CVs for solutions containing CD-DNA-Fc. In the HMFc experiments, square wave voltammetry (SWV) was performed to observe the redox reaction of 0.3 mM HMFc in the absence and presence of 15 mM β-CD in a 0.1 M $NaClO_4$ + 2.5 mM phosphate buffer solution (pH 7.0, Na^+ salt) using gold disc electrodes (BAS) (scanned from 0 to +0.5 V; step potential, 2 mV; amplitude, 25 mV; frequency, 50 Hz).

Results and discussion

IDA microelectrode chips are suitable for measuring the small electrochemical signals of small amounts of

electroactive species, with the advantages of enhancing the current responses due to the spherical diffusion of the electroactive species and due to the repeated regeneration of redox reacted species (*33–37*). In this study, IDA microelectrode chips were used to investigate the electrochemistry of small amounts of the probe CD-DNA-Fc solution. Cyclic voltammetry of the synthesised CD-DNA-Fc probe was performed using the carbon-based IDA microelectrode chips, with the potential at one electrode set at 0 V and the other electrode scanned at a scan rate of $0.01\,\mathrm{V\,s^{-1}}$. The CVs were corrected by subtracting a CV measured in a buffer solution as a baseline. The typical corrected CV of CD-DNA-Fc is demonstrated in Figure 1 (dashed line), showing that the anodic and cathodic waves of the Fc^0/Fc^+ redox reaction were sigmoidal, where the observed current intensity was increased at around +0.3 V and reached a plateau at more positive potentials. The diffusion-controlled limiting current at the anodic electrode was 18 nA. In general, IDA microelectrodes yield the theoretical equation for the diffusion-controlled limiting current, I_{lim}, in CVs as follows (*35*):

$$|I_{\mathrm{lim}}| = mbn\mathrm{Fc}^{*}D\left[0.637\ln\{2.55(1+w_{\mathrm{a}}/w_{\mathrm{g}})\} - 0.19/(1+w_{\mathrm{a}}/w_{\mathrm{g}})^{2}\right], \quad (1)$$

where m, b, w_{a} and w_{g} are the number, length, width and gap of the microband electrodes, respectively; n is the number of electron transferring in the redox reaction; F is Faraday's constant and $c*$ and D are the concentration and diffusion constant of the redox species, respectively. According to Equation (1), the diffusion coefficient of CD-DNA-Fc is induced to be $3.6 \times 10^{-7}\,\mathrm{cm^2\,s^{-1}}$. This diffusion coefficient is in agreement with those reported by other researchers for 20–29-mer oligonucleotides, from 10^{-7} to $10^{-6}\,\mathrm{cm^2\,s^{-1}}$ (*38–41*).

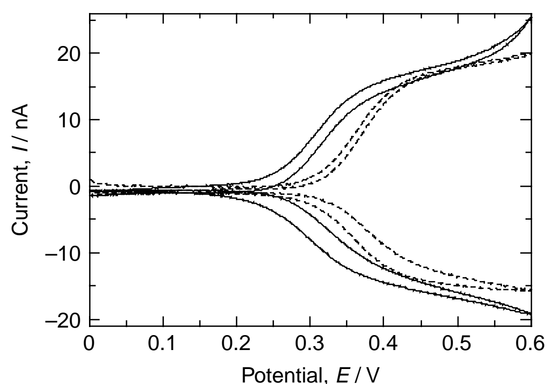

Figure 1. CVs for the redox reaction of 25 μM CD-DNA-Fc probe in a 0.075 M phosphate buffer solution (pH 7.0, Na^+ salt) + 0.0075 M NaCl in the absence (dashed line) and presence (solid line) of 25 μM target oligonucleotide using IDA chips at a scan rate of $0.01\,\mathrm{V\,s^{-1}}$.

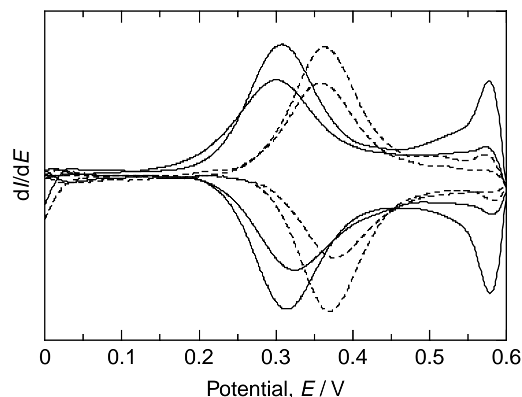

Figure 2. The value of dI/dE measured in the absence (dashed line) and presence (solid line) of the target oligonucleotide mentioned in Figure 1.

In the presence of the target oligonucleotide complementary to the probe, the anodic current was increased at around +0.25 V and reached a plateau at more positive potentials: the observed sigmoidal curve shifted towards a more negative potential without apparent change in the curve shape (solid line, Figure 1). In Figure 2, the curves of dI/dE vs. E of the CVs measured in the absence (dashed line) and presence (solid line) of the target oligonucleotide show that the inflection points of these CDs were +0.300 and +0.363 V, respectively, indicating that the potential shift was 62 mV. This potential shift of the redox reaction of the Fc moiety in CD-DNA-Fc is considered to be due to the formation and disassociation of the intramolecular inclusion complex between the Fc moiety and the β-CD moiety in CD-DNA-Fc. The limiting current at the anodic electrode in the presence of the target oligonucleotide was almost the same as that in the absence of the target, suggesting that the diffusion coefficients are not so much different between the single-stranded CD-DNA-Fc probe and the double-stranded CD-DNA-Fc/target hybrid. This fact also proved that the CD-DNA-Fc formed an intramolecular complex rather than an intermolecular complex, in the case of which aggregation of the probe and the target might provide a more reduced limiting current.

We demonstrated the dependence of the redox potential of a Fc derivative, HMFc, on complex formation with β-CD via SWVs using gold disc electrodes. In Figure 3, the SWV of 0.3 mM HMFc in the absence of β-CD in the solution showed a peak potential of +0.226 V (solid line), while the SWV in the presence of 15 mM β-CD showed a peak potential of +0.306 V (dashed line), showing a shift in the peak potential of 80 mV. The effects of CDs on the potential shift of the redox reaction of Fc or Fc derivatives have also been investigated by other researchers (*42–44*), confirming that the observed potential shift in this study was caused by the formation of an inclusion complex between the two terminal moieties in CD-DNA-Fc.

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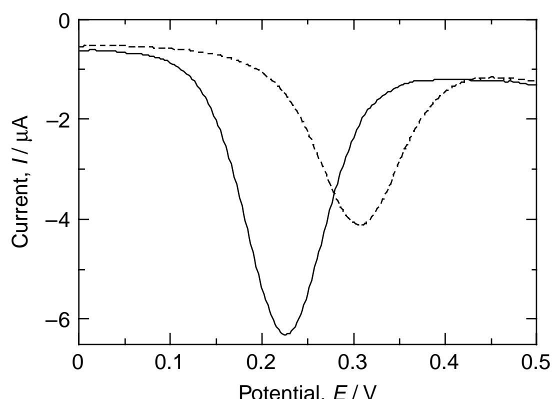

Figure 3. SWVs for the redox reaction of 0.3 mM HMFc in a 0.1 M $NaClO_4$ + 2.5 mM phosphate buffer solution (pH 7.0, Na^+ salt) in the absence (dashed line) and presence (solid line) of 15 mM β-CD using gold disc electrodes (scanned from 0 to +0.5 V; step potential, 2 mV; amplitude, 25 mV; frequency, 50 Hz).

A decrease in the peak current is also observed on the addition of β-CD (Figure 3). This is because the diffusion coefficient of the HMFc/β-CD complex is much smaller than that of HMFc and because the concentration of the HMFc/β-CD complex is larger than that of free HMFc in the solution.

Thus, the potential shift of the Fc redox reaction revealed hybridisation of the CD-DNA-Fc probe to the complementary target oligonucleotide, i.e. the probe detected the target with sequence specificity. Here, as shown in Figure 2, the current change was also observed before and after hybridisation. For example, while the current at +0.3 V was 1.31 nA in the absence of the target oligonucleotide, it increased 5.2-fold, reaching 6.91 nA, in the presence of the target, indicating that the CD-DNA-Fc probe provided a 'signal-on' response upon hybridisation. Previously reported 'signal-on' detection methods by other research groups require the use of stem structures or extra oligonucleotides to maintain complicated probe steric structures. This method is considerably simpler: the Fc and β-CD moieties act as the signal-generating and signal-suppressing parts, respectively, and the oligonucleotide strand in the probe simply acts as the target-recognition part. This probe design has the advantage over previous probe designs of allowing simple structures. Arbitrary sequences are available without any constraints on the design of probe sequences, while the relationship between the following two constants is important: the association constant between Fc and β-CD, and the stability constant between the probe and the target. The structure of the developed probe has signal-generating and signal-suppressing parts at both the terminals, making it similar to the structure of the so-called molecular beacons (*45–47*). However, there have so far been no reports on probes providing self-reporting 'signal-on' DNA detection based on the control of the hybridisation-sensitive redox signal by suppression and restoration of its own electrochemical activity. Although this study is conceptual and preliminary, the method proposed here has the potential for extension to a simple and rapid gene diagnostic method with the advantages of electrochemical techniques.

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Conclusion

In this study, the CD-DNA-Fc probe was designed to be terminated with a Fc and β-CD moieties at both the ends to act as signal-generating and signal-suppressing parts, respectively. Although the Fc and β-CD moieties formed an intramolecular inclusion complex in the absence of the target oligonucleotides, the complex was disassociated in the presence of the target due to the rigid structure of the double-stranded form, and the observed redox potential was negatively shifted by 62 mV and the observed current at +0.3 V increased more than 5-fold. The probe thus provided a 'signal-on' response upon hybridisation. Whereas this study is conceptual and preliminary, it is the first study on probes realising self-reporting 'signal-on' DNA detection based on the control of the hybridisation-sensitive redox signal by suppression and restoration of its own electrochemical activity.

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