Electrochemical Aberrant Methylation Detection Based on Ferrocenyl Naphthalene Diimide Carrying β-cyclodextrin, FNC

doi: https://doi.org/10.1002/elan.201900282

Electrochemical aberrant methylation detection based on ferrocenyl naphthalene diimide carrying β-cyclodextrin, FNC

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Received: ((will be filled in by the editorial sttaff))
Accepted: ((will be filled in by the editorial sttaff))

Abstract
Electrochemical detection of the specific gene carrying aberrant methylated cytosine was achieved by ferrocenyl naphthalene diimide carrying β-cyclodextrin (β-CD), FNC, coupled with the probe-DNA-immobilized electrode. The five CpG sites in a 24-base sequence were selected as the target DNA on the CDH4 gene, which is associated with colorectal cancer. When methylated and unmethylated samples hybridized with the DNA probe (HS-M24) immobilized on the electrode, an increased current signal was observed in the electrolyte containing FNC and correlated with the amount of target DNA. Furthermore, an increase in current (115%) was observed when the PCR product of 105 bp was hybridized on the HS-M24-immobilized electrode, whereas a background level of current increase was observed in the case of unmethylated product. Such large discrimination ability might be due to the inter- and/or intra-complex formation of ferrocene with β-CD of FNC on the surface of the electrode.

Keywords: aberrant methylation, cyclodextrin, electrochemical hybridization assay, ferrocene, naphthalene diimide derivative

DOI: 10.1002/elan.((will be filled in by the editorial sttaff))

1. Introduction

Gene detection has been important for the diagnosis of infectious diseases, cancer diagnosis, or prognosis and has become a crucial tool in influenza typing, or miRNA or methylation detection. Especially, methylation of cytosine in CpG is expected to be an effective marker for cancer diagnosis in the early stage and for prognosis assessment [1]. Detection of cytosine methylation has been reported with bisulfite sequence [2], methylation-specific PCR (MSP)[3], or COBRA[4]; these methods were developed as commercially-available kits, although they still have some limitations for an identifiable region. Recently, methylated cytosine detection based on fluorometric [5,6] or electrochemical techniques [7] has been developed to overcome the disadvantages.

Electrochemical techniques have an advantage in DNA detection, which was pioneered by Palecek [8] and its application in the detection of methylated cytosine has been reported; for example, methods based on the different redox potential of methylated cytosine and other nucleic acid bases [8-10], the enzymatic reaction of HRP-modified antibody for methylated cytosine coupled with

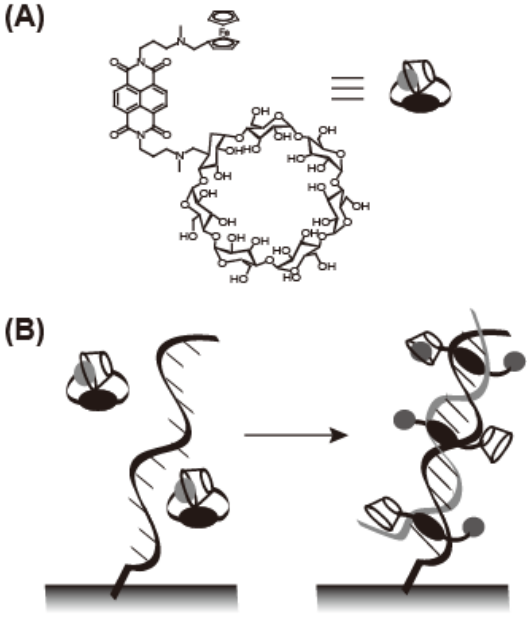

Fig. 1. (A) Chemical structure of FNC and (B) Strategy of an electrochemical hybridization assay by FNC.

H_2O_2/hydroxyquinone [11], or the electrochemical hybridization assay of MSP product using electroactive DNA ligands [12-17].

We have been developing an electrochemical methylation detection device using ferrocenyl naphthalene diimide (FND) derivatives as hybridization indicators for p53 [18], p16 [13], CDH4 [14], and hTERT genes [15,16] in combination with the MSP product after bisulfite treatment of the methylation-specific DNA probe immobilized on the gold electrode.

Here, electrochemical detection of aberrant methylation was carried out to improve the distinction between methylated and unmethylated samples using FND carrying β-cyclodextrin (β-CD) (FNC, Fig. 1A). We previously reported that inter- or intra-binding between ferrocene and β-CD of FNC binds to double-stranded DNA [19]. We have successfully detected PCR products using the potential changes in FNC in homogeneous solutions. This result is expected to be applicable for the detection of influenza virus and norovirus. As FNC should alter the complex stability with mismatched and fully matched hybrids, a large current change is expected, as shown in Figure 1B. To test this hypothesis, we tried to detect the methylation of CDH4 gene as hypermethylation of CDH4 gene has already been reported [20].

2. Experimental

2.1. Materials

The FNC was synthesized according to the procedure previously reported [19]. The custom synthesized oligonucleotide was purchased from Genenet (Fukuoka, Japan). Probe and sample DNA sequences are shown in Table 1.

Table 1. Probe DNA and target DNA sequences used in this experiment.

Probe	Sequence
HS-M24(+)	HO-$(CH_2)_6$-SS-$(CH_2)_6$-5'-ATG AT**C** G**C**G GG**C** GT**C** GG**C** GTG TTT-3'
M24(-)	3'-TAC TA**G** C**G**C CC**G** CA**G** CC**G** CAC AAA-5'
U24(-)	3'-TAC TAA CA**C** CC**A** CAA CC**A** CAC AAA-5'

PCR primers are as follows: MF-Primer, 5'–GTT TTC GGT GTC GGG TAT C-3', MR-Primer, 5'-CGA CAA CTT ACC CGA AAC G-3'. Template DNA for PCR was as follows: unmethylated template DNA, 5'-GAG CGG GTT TTC GGT GTC GGG TAT CGG GCG GGC GGC GGG GAA GAT GAT TGT GGG TGT TGG TGT GTT TTT GTT TTC GTT TTT CGG CGC GTT TCG GGT AAG TTG TCG TTT TTC-3', 5 methylated template DNA, 5'-GAG CGG GTT TTC GGT GTC GGG TAT CGG GCG GGC GGG GAA GAT CGC GGG CGT CGG CGT GTT TTT GTT TTC GTT TTT CGG CGC GTT TCG GGT AAG TTG TCG TTT TTC-3' (underlined parts are the target sequences). The 20× SSC buffer was purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). NaH_2PO_4, Na_2HPO_4, $NaClO_4$, and NaCl were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.2. PCR samples

The PCR product was obtained from 100 μL of PCR mixture of 1× Taq mix (Hot start plus, Qiagen GmbH, Hilden, Germany), 20 pM template DNA, 0.5 μM MF-Primer, and 0.5 μM MF-Primer after PCR amplification under the following conditions: 1 × 94 °C, 5 min; 45 × (94 °C, 15 s; 62 °C, 5 s; 72 °C, 10 s), and 1 × 72 °C, 10 s. The PCR product was purified by QIAquick PCR Purification Kit (Qiagen) and identified by the presence of gel band for 105 bp in native polyacrylamide gel electrophoresis.

Before the electrochemical measurement, various concentrations of the PCR product was prepared in 2× SSC, and it was incubated at 95 °C for 10 min for thermal denaturation. The PCR product was stored in a refrigerator until further use.

2.3. Electrochemical Hybridization Assay (EHA)

Electrochemical hybridization assay was carried out using a gold disc electrode (ϕ1.6 mm, BAS, West Lafayette, IN). Pretreatment of this electrode was carried out as per a previously reported procedure [14]; the electrode was dipped in 95 μL of 0.10 μM of DNA probe in 100 mM NaCl and incubated for 16 h at 37 °C for the detection of the methylated sequence. After washing with milli-Q water, the electrode was dipped in 95 μL of 1.0 mM 6-mercaptohexanol and incubated for 1 h at 45 °C. The sensor electrode was obtained after washing with milli-Q water. To achieve hybridization, the sensor electrode was dipped into 95 μL of 0.4 μM sample DNA in 2x SSC and incubated for 2 h at 15 °C.

Square wave voltammetric (SWV) measurements of the sensor electrode were carried out in an electrolyte containing 10 mM phosphate buffer (pH 7.0), 100 mM $NaClO_4$, and 2.5 μM FNC at 15 °C before and after hybridization. The SWV was conducted with Electrochemical Analyzer Model 650C (CH. Instruments) using the following conditions: Init E (V) = 0, Final E (V) = 0.6, Incr E (V) = 0.01, Amplitude (V) = 0.05, Frequency (Hz) = 10, Quiet Time (s) = 3, Sensitivity (A/V) = 1e-5.
For the detection of the PCR product, we used a custom-made multi-chip, which has 5 working, counter and reference electrodes (Tanaka Kikinzoku Kogyo K. K., Tokyo, Japan). Pretreatment of this chip was carried out using the procedure reported previously [15]. After

pretreatment, this chip was dipped in 1.0 μL of 0.10 μM probe DNA in 10% PEG200 (Wako) and 100 mM NaCl and incubated for 16 h at 37 °C. PEG200 was used to prevent the drying of the probe DNA solution during the immobilization process. After washing with milli-Q water, the chip was dipped in 400 μL of 1.0 mM 2-mercaptoethanol and incubated for 1 h at 45 °C. Hybridization was achieved by dipping 1.0 μL of the PCR product in 2× SSC, and then, keeping it undisturbed for 2 h at 15 °C. Square wave voltammetric measurement of this chip was carried out in 10 mM phosphate buffer (pH 7.0) containing 100 mM $NaClO_4$ and 2.5 μM FNC using Electrochemical Analyzer Model 650C, under the conditions mentioned above. All results were evaluated with $\Delta i = (i/i_0 - 1) \times 100$, where i_0 and i are peak currents before and after hybridization, respectively.

3. Results & Discussion

3.1. Electrochemical detection of target oligonucleotide with FNC

The thiolated oligonucleotide, which is a part of CDH4 gene (HS-M24(+), Table 1), was utilized as a DNA probe. Probe-DNA-immobilized electrode was prepared by treating with 0.10 μM HS-M24(+) in 100 mM NaCl and hybridizing with 0.40 μM M24(-) in 2× SSC. The probe DNA and target DNA were designed such that the fully matched and mismatched base pairs were C-G and C-A, respectively, to expand the difference between their thermal stabilities [14]. Optimized conditions were utilized, as per the previous reports for the probe DNA immobilization [14], 6-mercaptohexanol masking [21], electrolyte [19], and hybridization [14]. Frequency dependence was measured in 10 mM phosphate buffer (pH 7.0), and 100 mM $NaClO_4$ containing 0.5, 2.5, or 5.0 μM FNC, using SWV measurement (Fig. S1). The current difference before and after hybridization was maximized under 10 Hz, even under varied FNC concentrations. This result is in agreement with the fact that the largest current at 10 Hz was observed with ferrocenyl double-stranded DNA on the electrode than that with ferrocenyl single-stranded DNA or mismatched double-stranded DNA [22]. In other words, DNA duplex bound to FNC on the electrode exhibited behavior similar to the ferrocenyl oligonucleotide. However, the obtained current increases

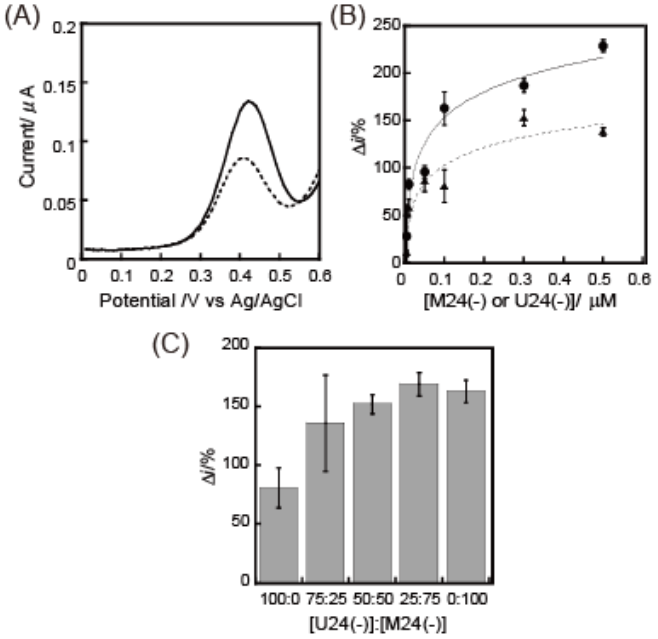

Fig. 2. (A) SWV results of before (dotted line) and after hybridization (solid line) of M24(-). (B) Concentration dependence of the Δ*i* value for M24(-) (●) or U24(-) (▲). (C) The Δ*i* value after hybridization of the mixtures of U24(-) and M24(-). All experiments were carried out using HS-M24(+)-immobilized electrode in 10 mM phosphate buffer (pH 7.0) and 100 mM $NaClO_4$ containing 2.5 μM FNC.

the contribution of the diffusion current with an increase in FNC concentration, and the current difference between single-and double-stranded DNAs was diminished.

Figure 2A shows the square wave voltammogram before and after hybridization with complementary target DNA in 10 mM phosphate buffer (pH 7.0), and 100 mM $NaClO_4$ containing 2.5 μM FNC. Before hybridization, it is presumed that a current was observed in which a slight diffusion current component of FNC and an electrostatic adsorption component of FNC on probe DNA were combined. The peak current was not shifted before and after hybridization. In our previous research on FNC, the current change before and after binding to the DNA duplex was by intercalation, which is due to the change in the environment of the ferrocene in FNC before and after hybridization; ferrocene of FNC forms an intra-molecular inclusion complex in an aqueous medium. The complex collapses after binding to a double-stranded DNA, resulting in the negative shift of redox potential of FNC in a homogenous medium [23]. However, the current shift was not observed in the case of the DNA-immobilized electrode before and after hybridization. This suggested that FNC maintains an inclusion complex after hybridization, suggesting the formation of an inclusion complex of FNC bound to double-stranded DNA by intercalation. This might form the same DNA duplex strand or might get cross-linked with a neighboring DNA

duplex strand, as shown in Fig. 3. Similar behavior was observed under atomic force microscopy (AFM) for the complex of FNC and double-stranded DNA under specific conditions [19].

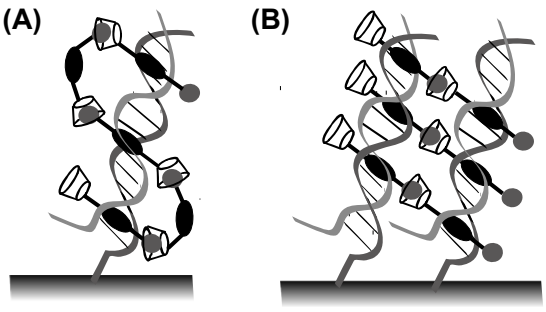

Fig. 3. Expected complex cartoon of FNC with the same DNA duplex strand (A) or cross-linking neighboring DNA duplex strand (B) on the electrode.

As a next step, the concentration dependence of the sample DNA was studied after hybridization with 0.005, 0.01, 0.05, 0.1, 0.3, 0.5 μM of M24(-) or U24(-) with HS-M24(+)-immobilized electrode. Figure 2(B) shows that peak current increased with an increase in the concentration of M24(-) as the target DNA and reached up to 200 % of Δi, whereas 150% of Δi was observed in the case of mismatched one of U24(-). The density of the DNA probe on the electrode was estimated as 1×10^{12} molecules/cm^2 from chronocoulometry (CC) measurement (data not shown). At such density of DNA probe-immobilized electrode, the current increase for fully matched or mismatched DNAs was around 80%, and 60% in the case of ferroceneyl naphthalene diimide (FND) [14]. Compared to the previous result, the increase in current in case of FNC was higher than that in case of the previous one.

3.2. Detection of a mixed sample of methylated and unmethylated DNA

When the methylated gene was detected from any clinical sample, it was always contaminated with an unmethylated sample, and thus, it is important to detect methylated sample under the mixture of unmethylated one. Then, sample was prepared with 0.10 μM of the mixture having the following ratio: U24(-):M24(-) = 100:0, 75:25, 50:50, 25:75, and 40:100. Figure 2C shows the peak current after hybridization of the above sample with HS-M24(+)-immobilized electrode. An increase in current was observed with the increasing content of target M24(-), suggesting the possibility of target DNA containing similar DNA sample. The current increase of 136±41% was observed under 25% (0.25 μM) of M24(-), and almost 100% was obtained at > 75% presence of M24(-), which has values same as that of 100% M24(-).

3.3. Detection of PCR product

The PCR products, M105 and U105, were prepared as part of the CDH4 gene. Target 24-bp was designed to locate the middle of 105-mer in these PCR products [14]. The U105 was hybridized with the HS-M24(+)-immobilized electrode at varied concentrations of 0.30, 0.75, 1.5, 2.3, 3.0, 4.0, 5.0, or 7.5 ng/μL in 2× SSC.

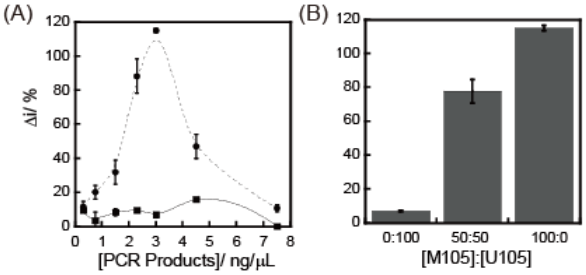

Fig. 4. (A) The Δi value upon hybridization with 0.005-5.0 ng/μL of M105 (●) or U105 (▲). (B) the Δi value after hybridization of the mixed sample of U105 and M105. Both experiments were carried out using HS-M24(+)-immobilized electrode in 10 mM phosphate buffer (pH 7.0) and 100 mM $NaClO_4$ containing 2.5 μM FNC.

Figure 4(A) shows the Δi value against the concentration of U105. The Δi value increased with an increase in the concentration of U105 from 0.3 to 3.0 ng/μL and reached 115% maximum value. However, the Δi value decreased at 4.5 ng/μL. In the case of M105, as it is methylated, a small current increase, within 29%, resulted in the discrimination of methylated and unmethylated PCR products with high efficiency. We estimated the hybridization efficiency of these PCR products and showed very low efficiency; 20% for the fully matched combination and 15% for the mismatched one, whereas the hybridization efficiency for this oligonucleotide was 80% for the fully matched combination and 60% for mismatched one [14]. These results suggested that FNC expands the discrimination ability between fully matched and mismatched DNA samples. The reason for the decreasing Δi value beyond 4.5 ng/μL of U105 might be due to the formation of aggregation of the PCR products with FNC [19].

The FND response tends to decrease when there are more PCR products to hybridize [24]. It is thought that intercalators cannot bind or they may inhibit electron transfer of ferrocene because of the crowding on the electrodes. In Figure 4A, a large current decrease was observed upon treatment with ≥4.5 ng/μL PCR products. As DNA is aggregated by FNC [23], DNA is more likely

to be aggregated on the electrode, and electron transfer of ferrocene would be inhibited.

Furthermore, when 3.0 ng/μL of mixed PCR products of U105:M105 = 100:0, 50:50, or 0:100 were hybridized with HS-M24(+)-immobilized electrode, the Δ*i* value increased with increase in in the content of target M105 (Fig. 4(B)).

4. Conclusion

Methylation-specific PCR products were detected using the electrochemical hybridization assay coupled with DNA probe-immobilized electrode and FNC. Under optimum conditions, perfect distinction between methylated and unmethylated PCR product was possible because FNC acts as the effective stabilizer of the DNA duplex on the electrode. We have previously successfully detected PCR products in homogeneous solution with FNC, which allows detection of PCR products; for example, influenza typing. In addition to the detection of methylation, it is also applicable to miRNA detection and SNP detection through DNA chips coupled to FNC. Although methylation detection and SNP detection require PCR, miRNA may be directly detected without PCR using this system.

5. Acknowledgments

This study was performed as a research program of Ministry of Education, Culture, Sports, Science and Technology of Japan. Furthermore, this work was supported in part by JSPS KAKENHI, and Grant-in-Aid for Scientific Research (C) Grant Number 26463074.

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