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Electrochemical detection of aberrant methylated gene using naphthalene diimide derivative carrying four ferrocene moieties

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ABSTRACT

Naphthalene diimide derivative, F4ND, carrying four ferrocene moieties was synthesized as a new hybridization indicator for electrochemical gene detection using DNA probe-immobilized electrode. F4ND could bind to double stranded DNA with threading intercalation mode despite the existence of such bulky substituents. As result, an improved binding affinity due to slower dissociation from double stranded DNA was observed. F4ND could be used for electrochemical discrimination between methylated and unmethylated PCR product of CDH4 gene coupled with methylation specific DNA probe-immobilized electrode under the optimum condition.

1. Introduction

Gene detection using electrochemical technique is expected to offer next generation of biochips [1]. So far many techniques including label-based or label-free methods [2,3] have been reported for this purpose. Label-based methods can be classified into three categories. First approach in these categories is to attach an electrochemical signal molecule to a sample DNA [4–6], which participates in a sandwich hybridization assay [7–9]. Electrochemically-active DNA sample is allowed to hybridize with the DNA probe-immobilized electrode and the amount of the target DNA is estimated by the electrochemical signal generated by the DNA sample remained on the electrode. Second group of methods exploits the conformation change of the DNA probe, carrying electrochemically-active molecule immobilized on the electrode, after hybridization with target DNA sample [10]. Hairpin structure of this DNA probe on the electrode is a typical example and this structure, which is destroyed by the hybridization with target DNA. The electrochemical signal change is observed depending on the distance between electrochemically-active molecule and the electrode surface [11]. However, these two systems have disadvantages for PCR products such as difficulty in their labeling with electrochemically-active molecule or decreasing the hybridization efficiency between short DNA probe and PCR product being a long DNA sample.

Third approach exploits electrochemically-active DNA ligands, especially having a banding preference for double stranded DNA [12–14]. Intact sample DNA is allowed to hybridize with DNA probe-immobilized electrode and the information on hybridization efficiency or amount of target DNA is estimated from the DNA ligand bound to double stranded DNA region on the electrode. This system is useful when considering the absence of any labeling steps of DNA probe and DNA sample. When PCR products are used as DNA sample, this system might work well because the hybridization proceeds between intact DNAs and stabilization of the formed double stranded DNA by the binding of DNA ligand is expected. However, DNA ligand is required to have high preference for double stranded DNA over single stranded DNA. Therefore, we have been developing naphthalene diimide derivative having ferrocene moieties at its both termini [15–23] and applied this ligand to the detection of PCR product [17,18,21,22].

In this paper, naphthalene diimide having two ferrocene moieties at both termini (F4ND, Fig. 1) was designed and synthesized to evaluate the feasibility of electrochemical detection of PCR product derived from aberrant methylation of CDH4, which is concerned with colon cancer [24].

2. Experimental section

2.1. General

Calf thymus DNA was purchased from Sigma–Aldrich (St. Louis, MO) and used after sonication according to the method reported

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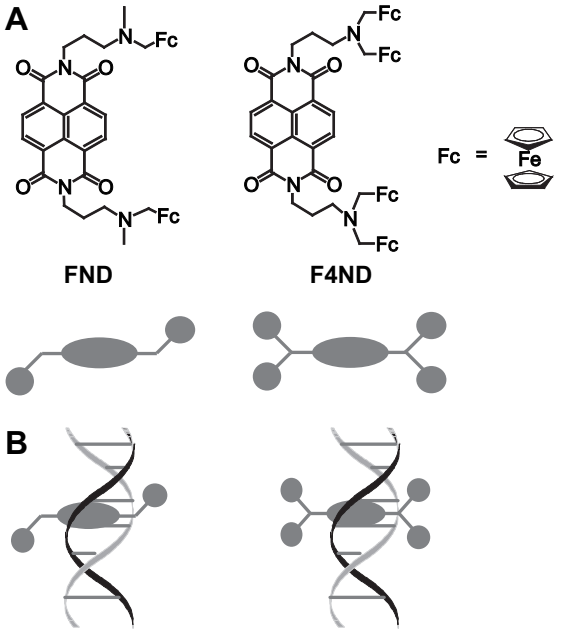
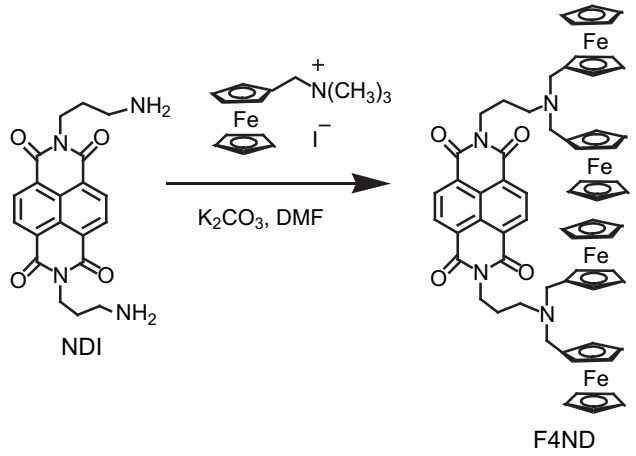


Fig. 1. (A) Chemical structures of F4ND and FND and (B) the expected structure of their complexes with DNA duplex.



Scheme 1.

previously [25]. The concentration of calf thymus DNA was estimated from the molar absorptivity of 6412 $cm^{-1}\,M^{-1}$ at 260 nm based on nucleic bases [26]. Oligonucleotides used in this study were custom synthesized by Genenet Co. (Fukuoka, Japan) and their concentrations were estimated from the molar absorptivity values as shown in Table 1. PCR product was prepared with 150 mer oligonucleotide as template. Buffer composed of 10 mM MES (pH 6.2), 1 mM EDTA, and 0.10 M NaCl was used in viscosity titration, and kinetic experiment. UV–Vis absorption titration experiments were carried out in 10 mM AcOK–AcOH (pH 5.5), 0.10 M KCl at 25 °C. Melting studies of DNA duplex in the absence or the presence of FND or F4ND were 20 mM AcOK–AcOH (pH 5.5), 20 mM KCl. The composition of 2 × SSC as a hybridization buffer in the electrochemical experiments was 0.03 M sodium citrate and 0.30 M NaCl. Electrolyte used in electrochemical experiments was 0.10 M AcOK–AcOH buffer (pH 5.5) and 0.10 M KCl containing 5.0 μM of F4ND.

2.2. Apparatus

Mass spectra (MS) were taken on a Voyager™ Linear-SA (PerSeptive Biosystems, Foster City, CA) by the time-of-flight mode with α-cyano-4-hydroxycinnamic acid as matrix. Viscosity titrations were carried out with a PC-controlled automatic system (Lauda, Lauda-Königshofen, Germany) equipped with a capillary Ubbelohde-type viscometer, an automatic pump/stop-watch unit and a thermostatted water bath at 30.0 ± 0.1 °C. Electronic absorption spectra were recorded with a Hitachi 3300 spectrophotometer equipped with an SPR 10 temperature controller. Kinetic experiments were performed with an SF-61DX2 double mixing stopped flow system (Hi-Tech Scientific, Salisbury, UK) equipped with a Lauda RF206 temperature controller. PCR was carried out using Program temp control system PC-320 (Astec Co., Ltd, Fukuoka, Japan). Electrochemical measurements were carried out with an ALS model 600 electrochemical analyzer (CH Instrument, Austin, TX).

2.3. Synthesis of N,N-bis(3,3′-diferrocenylmethylaminopropyl) naphthalene-1,4,5,8-tetracarboxylic acid diimide

The synthetic route of F4ND is shown in Scheme 1. A solution of (ferrocenylmethyl)trimethylammonium iodide (3.0 g, 26 mmol), N, N′-bis(3-aminopropyl)naphthalene-1,4,5,8-tetracarboxylic acid diimide [19,23] (0.5 g, 1.3 mmol), and K_2CO_3 (3.4 g, 26 mmol) in DMF (50 mL) was stirred at 90 °C for 18 h. After cooling to room temperature, the mixture was filtrated and the filtrate was distilled away under reduced pressure. The residue was dissolved in $CHCl_3$ and washed 4 times with saturated sodium bicarbonate solution. The solution was evaporated and the residue was chromatographed on silica gel (Merck 60) with the eluent consisting of THF: hexane = 2:5. The fraction showing an Rf of 0.1 on TLC (THF: hexane = 2:5) was collected and the solvent was removed under reduced pressure. The desired compound F4ND was obtained as a dark green solid (0.28 g, 18%). m.p. 221–223 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.82 (m, 4H, CH_2), 2.39 (m, 4H, CH_2), 3.36 (s, 8H, N–CH_2–Fc), 3.97–4.26 (m, 40H, Fc–H, CH_2), 8.68 (s, 4H, Ar-H) ppm; Elemental Anal. Calc. for $C_{64}H_{60}N_4O_4Fe_4$; C, 65.56; H, 5.16; N, 4.78. Found: C, 65.64; H, 5.36; N, 4.89%. MS m/z $[M+H]^+$ 1174.3 (calcd for $C_{64}H_{60}N_4O_4Fe_4 + H^+$ 1173.5).

2.4. Binding studies

The binding affinity of F4ND for sonicated calf thymus DNA was determined by Scatchard analysis reported previously [27] to obtain observed binding constant, K, with site size, n, as the number of base

Table 1
DNA sequences used in this experiment.

	Sequence	ε_{260nm} ($cm^{-1}\,M^{-1}$)
HS-M24(+)	5′-SH-ATG ATC GCG GGC GTC GGC GTG TTT-3′	222,800
M24(−)	3′-TAC TAG CGC CCG CAG CCG CAC AAA-5′	226,000
U24(−)	3′-TAC TAA CAC CCA CAA CCA CAC AAA-5′	238,900
M105(−)	3′-(37mer)-TAC TAG CGC CCG CAG CCG CAC AAA-(43mer)-5′	
U105(−)	3′-(37mer)-TAC TAA CAC CCA CAA CCA CAC AAA -(43mer)-5′	

pairs excluded by the bound ligand. Kinetic experiments were performed according to procedure previously described [28,29] and obtained association rate constant, k_a, and dissociation rate constant, k_d in the binding system of F4ND with the DNA were calculated.

2.5. Preparation of PCR product

In methylated (mutant-type) and unmethylated gene (wild-type), the methylated and unmethylated cytosine bases were converted to cytosine and uracil, respectively, by the bisulfite treatment and such modified DNA can be subsequently amplified using PCR. Two following oligonucleotides (having the same primer sequences to allow PCR using same primers) were used as templates for methylated and unmethylated types after bisulfite treatment in this experiment. Mutant-type (methylated type, M105(+)): 5′-GAG **C**GG GTT TT**C** GGT GT**C** GGG TAT **C**GG G**C**G GG**C** GG**C** GGG GAA GAT GAT **C**G**C** GGG **C**GT **C**GG **C**GT GTT TTT TTT GTT GTT TT**C** GTT TTT **C**GG **C**G**C** GTT T**C**G GGT AAG TTG T**C**G TTT TT**C**-3′, Wild-type (unmethylated type, U105(+)): 5′-GAG **T**GG GTT TT**C** GGT GT**C** GGG TAT **C**GG G**T**G GG**T** G**T**T GGG GAA GAT GAT **T**G**T** GGG **T**GT **T**GG **T**GT GTT TTT TTT GTT GTT TT**T** GTT TTT **T**GG **T**G**C** GTT T**C**G GGT AAG TTG T**C**G TTT TT**T**-3′. Underlined DNA sequences show the PCR primer sites and underlined single bases indicate the mutation sites: Forward primer; 5′-GTT TTC GGT GTC GGG TAT C-3′, Reverse primer; 5′-CGA CAA CTT ACC CGA AAC G-3′. One hundred μL of the reaction solution of asymmetric PCR was prepared from the following mixture: 50 μL of PrimeSTAR® (Takara Bio Inc., Shiga, Japan, PrimeSTAR HS DNA polymerase 1.25 units 25 μL^{-1}, 2 × dNTP Mixture each 0.4 mM, and 2 × PrimeSTAR Buffer 2 mM Mg^{2+}), 40 pM of template for mutant- or wild-type, 0.4 μM R primer, and 4 nM F primer was used for PCR by 45-times repeating the following cycle: 94 °C for 15 s, 62 °C for 5 s, 72 °C for 10 s and 72 °C for 10 min as final elongation. Sample was pre-heated at 94 °C for 5 min. After asymmetric PCR, product, which partially contained single stranded strand, was recovered by Micropure EZ and Microcon YM-30 (Millipore, Billerica, MA).

2.6. Preparation of the DNA probe-immobilized electrode

A gold electrode (0.02 cm^2 in area) was purchased from Bioanalytical Systems Inc. (Tokyo, Japan) and was polished with 6 μm, 1 μm of a diamond slurry, 0.05 μm of an alumina slurry in order each for 10 min and then washed with Milli-Q water 5-times each for 3 min. The electrode was electrochemically polished by scanning 40 segments from −0.2 to 1.5 V at a scan rate of 100 mV s^{-1} in 0.50 M H_2SO_4 aqueous solution and washed with Milli-Q water 5-times each for 3 min. HS-M24(+) electrode as a DNA probe-immobilized electrode was prepared by soaking in 95 μL of 0.20 μM HS-M24(+) containing 1.0 M NaCl, keeping at 37 °C for 16 h, washing with Milli-Q water, and soaking in 95 μL of 1.0 mM 6-mercapthexanol (MCH) aqueous solution at 45 °C for 1 h.

2.7. Hybridization

Hybridizations with sample DNAs were carried out by soaking the HS-M24(+) electrode in 95 μL of 0.50 μM sample oligonucleotides in 2 × SSC or 48 μL of PCR product for 2 h at the proper temperature.

2.8. Electrochemical measurements

After washing the HS-M24(+) electrode with Milli-Q water, the electrode was soaked in the electrolyte at the proper temperature for 2 min and CV measurements were carried out with a three-electrode configuration consisting of an Ag/AgCl reference electrode, a Pt counter electrode and the HS-M24(+) electrode as working electrode before and after hybridization with scan rate of 100–700 mV s^{-1}, sample interval of 1 mV, and sensitivity 1×10^{-6} A/V. Square wave voltammetry (SWV) was also measured with a three-electrode configuration with applied potential of 10 mV, amplitude of 50 mV, frequency of 250 Hz, and sensitivity 1×10^{-5} A/V. All data were standardized using Δi values, defined as $(i/i_o - 1) \times 100\%$, where i_o and i refer to the current before and after hybridization, respectively.

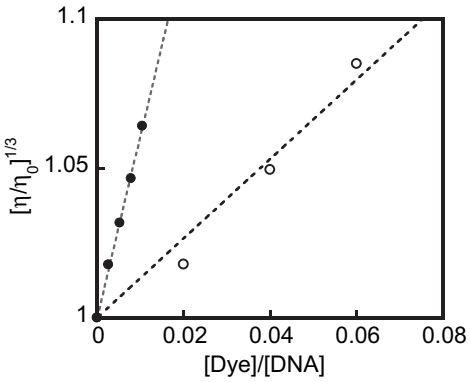


Fig. 2. Viscometric titration of 50 μM $[poly(dA–dT)]_2$ with various amounts of F4ND (●) and FND (○) in 10 mM MES and 1 mM EDTA (pH 6.25) containing 0.10 M NaCl at 30 °C.

3. Results

3.1. Binding behavior of F4ND with double stranded DNA

Absorption spectra of F4ND showed an absorption maximum at 383 nm in MES buffer and hypochromicity of 56% and red shift of 2 nm were observed upon addition of sonicated calf thymus DNA, which is in agreement with those for FND [19]. Threading intercalation of F4ND into double stranded DNA was confirmed by viscosity of a DNA solution that increased linearly with increase in the amount of F4ND and the slope for F4ND was larger than that for FND [19] as shown in Fig. 2. The viscosity increase is known to be caused not only by the unwinding of DNA duplex due to the intercalator, but also is affected by the substituent size of the intercalator or by the distortion of DNA structure after binding of the intercalator [30]. Therefore, this result suggested that the bulky substituents of F4ND caused the large unwinding of DNA duplex. The binding constant of F4ND with calf thymus DNA, calculated from McGhee & von Hippel-type Scatchard equation, was found to be of 1.1×10^6 M^{-1} with n value close to 2 as shown in Table 2. This binding constant is ca. ten-times larger than that of FND [19]. To estimate F4ND binding preferences towards single and double stranded DNA, the UV–Vis absorption titrations were carried out using 22-meric single or double stranded oligonucleotides. However, no absorption change was observed upon addition of single stranded one whereas large hypochromic effect and red shift were observed in the case of double stranded DNA (Fig. S1). Melting temperatures of 2 μM-strand(s) of $(dA)_{22}\cdot(dT)_{22}$ were also measured

Table 2
Binding (K, n) and kinetic parameters for naphthalene diimide derivative/sonicated calf thymus DNA systems.

Ligand	10^{-5} K (M^{-1})	n	10^{-4} k_a (M^{-1} s^{-1})	k_d (s^{-1})
F4ND[a]	11.2	1.8	12.2	0.57
FND[b]	1.0	2.7	14.1	1.34

[a] Experiment was carried out in 10 mM MES, 1.0 mM EDTA (pH 6.2), and 0.10 M NaCl.
[b] Ref. [19].

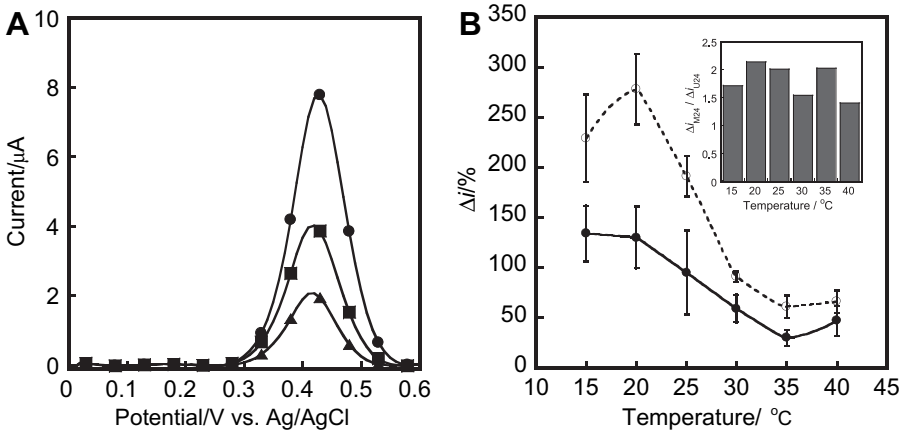


Fig. 3. (A) Typical example of the square wave voltammograms of HS-M24(+)-immobilized electrode before (▲) and after hybridization with M24(−) (●) or U24 (■) in 0.10 M AcOH–AcOK (pH 5.5), 0.10 M KCl, and 5.0 μM F4ND at 20 °C. (B) Temperature dependence of Δ*i* value in the case of fully matched (broken line) or mismatched combination (solid line).

in the absence or the presence of 5 μM F4ND or FND in 20 mM AcOK–AcOH (pH 5.5) and 20 mM KCl. Both ligands could stabilize double stranded structure having $T_m = 38$ °C in the absence of ligands by $\Delta T_m = 4$ °C without particular stabization preferences as shown in Fig. S2 and Table S1. This result suggests that F4ND may distort duplex structure upon binding to double stranded DNA. This is reasonable when considering its bulky substituents and literature reports on DNA bending intercalators [20]. Kinetic experiments were also carried out and the kinetic parameters for the binding of F4ND with sonicated calf thymus DNA are summarized in Table 2. The dissociation rate constant of F4ND with calf thymus DNA is ca. two-times smaller than that of FND whereas the association rate constant is almost the same as that for FND. These results suggest that F4ND can bind to double stranded DNA with a threading intercalation mode, despite that such bulky substituents of F4ND require to be projected out over both DNA grooves.

3.2. Electrochemical behavior of F4ND

Cyclic voltammograms for the HS-M24(+)-immobilized electrode before and after hybridization with its complementary oligonucleotide were measured in the electrolyte and corrected with the response for the electrode treated with MCH alone. Electrochemical response based on F4ND was observed in the case of HS-M24 (+)-immobilized electrode, whereas flat line was observed in the case of MCH-treated electrode. The oxidation at $E_{ap} = 435$ mV and the reduction at $E_{cp} = 409$ mV (rate of potential scan, $v = 100$ mV s^{-1}), giving $E_{1/2} = 422$ mV were observed in the case of F4ND. $E_{1/2}$ value of F4ND is negatively shifted comparing with that of FND ($E_{ap} = 422$ mV, $E_{cp} = 411$ mV, $E_{1/2} = 430$ mV) [15,21].

3.3. Electrochemical detection of target DNA and discrimination of mismatched one using F4ND

Sequence of HS-M24(+) was designed as 24-mer positive strand of exon 1 on methylated CDH4 gene to easily discriminate between methylated and unmethylated DNA samples after bisulfite and PCR treatment, where unstable C–A mismatch was formed by the unmethylated sample [31]. Stability of duplexes of HS-M24(+) with U24(−) or M24(−) was tested by melting experiments in 0.1 M AcOH–AcOK (pH 5.5) and 0.1 M KCl. T_m value of the mismatched combination of M24(+) and U24(−) (MM duplex) was 54 °C indicating formation of stable duplex in room temperature, whereas the T_m value for the perfectly matched combination (PM duplex) was 79 °C (Table S2). The relatively high stability of the mismatched DNA duplex might derive from its high GC content and feasibility of hydrogen bonding formation between A and C nucleobases [32]. Hybridization of the HS-M24(+)-immobilized electrode was carried out with U24(−) or M24(−) sample oligonucleotide in the varied hybridization temperature from 15 to 40 °C. SWV measurements were conducted in the same temperature as in the

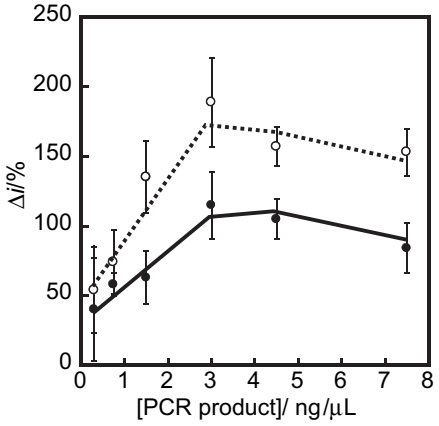


Fig. 4. Concentration dependence of the relative current (Δ*i*) for hybridized M105(−) (broken line) or U105(−) (solid line) asymmetric PCR products with HS-M24 (+)-immobilized electrode.

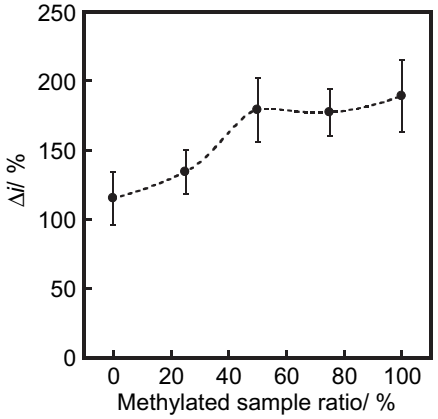


Fig. 5. Plot of Δ*i* value against the mixture of M105(−) and U105(−) with the varied ratio.

case of its hybridization. Square wave voltammograms and the temperature dependences of Δi value in the case of methylated and unmethylated combination were plotted in Fig. 3(A) and (B). Fully matched combination between HS-M24(+) and M24(−) showed the larger Δi value than mismatched combination between HS-M24 (+) and U24(−) under all of the temperature values and the largest discrimination ($\Delta i_{M24}/\Delta i_{U24} = 2.1$) was observed at 20 °C as shown in Fig. 3B. Thus, the experiment using PCR product was carried out at 20 °C (both hybridization and SWV measurement).

Asymmetric PCR product of methylated (M105(+)) or unmethylated (U105(+)) sample was used for this detection system. Fig. 4 showed the concentration effect of PCR product on the Δi plots. The Δi values in the case of M105(−) and U105(−) increased linearly up to 3.0 ng/μL and reached plateau, in which the Δi value of fully matched or mismatched case showed 150 or 100%, respectively. This result showed that the methylation detection of this gene can be achieved when used 48 μL DNA sample containing up to 3.0 ng/μL concentration of DNAs. Mixtures of M105(−) and U105(−) with varied ratios were also tested by this method and Δi value increased up to 60% of U105(−) and reached plateau as shown in Fig. 5. This result suggested the possibility of quantification of M105(−) in the sample DNA mixture.

4. Discussion

F4ND having two ferrocene moieties at its both termini was designed to show the improved stabilization of the complex with double stranded DNA to enhanced electrochemical signal. Although F4ND has too bulky substituents, it could bind to double stranded DNA with threading intercalation mode, which was confirmed by the absorption and viscometric titration experiments. Dissociation of F4ND from double stranded DNA became slower than that of FND suggesting the contribution of its substituents as effective anchors. This result suggested that DNA duplex in an aqueous solution is flexible enough to accept larger substituent than the space between adjacent base pairs. When considering the substituent size of F4ND, the base pair opening of DNA duplex should contribute upon formation of intercalation complex.

Electrochemical measurements could be carried out at 5.0 μM F4ND, where significant electrochemical signal was not observed in the electrode covered with MCH alone. Such low concentration of F4ND could reduce the electrochemical signal based on diffusion of F4ND from bulk solution. The discrimination ability between fully matched and mismatched combinations was improved by F4ND because of its improved binding affinity and increased number of ferrocene moieties per one ligand molecule. In the reported case, the DNA hybrid on the electrode possesses a 24-base-pair double stranded region and two single-stranded overhangs: of 37 and 43 bases, projecting in opposite directions on the electrode surface. The single-stranded overhangs may diminish hybridization efficiency. In fact, other papers reported the low hybridization efficiency in such conditions [33,34]. The relatively good hybridization efficiency achieved in our case comes probably from the stabilization effect of F4ND threading intercalation on double stranded DNA. This ligand could stabilize DNA duplex and the stabilization extent of fully matched combination was higher than that of mismatched combination, which is in agreement with the case of FND [35]. The Δi values obtained in this experiment allowed discrimination between fully matched and mismatched combinations within the error, although, scattered results were observed for the individual electrodes. This comes from the variations in the immobilized amount of DNA probe on the electrode. When the amount of DNA probe on the electrode is controlled, this system will work well for a variety of PCR products.

5. Conclusion

Naphthalene diimide derivative having four ferrocene moieties, F4ND, was synthesized and showed the threading intercalation with double stranded DNA. This ligand could improve the discrimination ability between fully matched and mismatched combination in the electrochemical detection system. The result obtained here will have a potential to design the electrochemical hybridization indicator with an improved performance.

Acknowledgements

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2010.04.034.

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