Naphthalene diimide carrying four ferrocenyl substitutes as an electrochemical indicator of tetraplex DNA aiming at cancer diagnosis

Shinobu Sato a,b, Atsuhito Kajima a, Hisashi Hamanaka a, Shigeori Takenaka a,b, *

a Department of Applied Chemistry, Kyushu Institute of Technology, Fukuoka, 804-8550, Japan
b Research Center for Bio-microsensing Technology, Kyushu Institute of Technology, Fukuoka, 804-8550, Japan

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A B S T R A C T
Naphthalene diimide carrying four ferrocenyl substituents, tFND1 and tFND2, were newly synthesized to expect the improved binding affinity for tetraplex DNA and specific electrochemical signal generation. tFND2 shows the 1:2 binding to tetraplex A-core DNA, which has a part of human telomere sequence based on circular dichroism spectra, and the current increase of tFND2 on the tetraplex DNA-immobilized electrode was 13.5-times higher than that on the single stranded DNA-immobilized electrode. When applied to an electrochemical telomerase assay (ECTA) for HeLa cell, tFND2 shows the quantitative change around 0.1–50 cells/µL and the detection limit of 0.1 cells/µL for HeLa cell.

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1. Introduction

Oral cancer is known to show a mild pain or uncomfortable feeling in an early phase and to be found out in a progress stage. Treatment under the progress stage causes a bottleneck in eating, talking, and breathing, resulting in a decline in the quality of life. Under such circumstances, it is important to develop the oral cancer diagnosis method under the early stage and especially a simple and rapid diagnostic method desired because of the absence of distinctive symptom under the early stage. Many researchers have been focusing on a telomerase activity, which is known to act in over 80% cancer cell even in the early stage [1].

Conventional telomerase detection has hitherto been carried out with the telomerase repeating amplification protocol (TRAP), which is based on the observation of band length of the telomere DNA fragments elongated from a telomerase substrate (TS)-primer with telomerase under gel electrophoresis [2]. This TRAP requires a PCR and the gel electrophoresis, which contains a tedious and time-consuming procedures. Our developing method is based on an electrochemical method using TS-primer-immobilized electrode and electrochemically active tetraplex DNA ligand. Other groups also developed the electrochemical telomerase assay [3–7]. Shao et al. achieved from the oxidation current of guanine (G) of the elongated telomere DNA on the electrode [3]. They utilized the ITO electrode because of its large potential window to detect 1.0 V of the oxidation voltage of G. The detection limit of this method was 50 ng/µL of telomerase [3]. Lin et al. also developed telomerase detection using the methylene blue-modified hairpin DNA probe carrying a complementary sequence of telomere DNA (MB-HP DNA), TS-primer, and T7 exonuclease [4]. Telomerase elongated TS-primer and hybridized with many MB-HP DNAs, which are correlated the length of the elongated TS-primer to digest with T7 exonuclease resulting in many mononucleotides having methylene blue. These mononucleotides showed a reduction on the ITO electrode. Telomerase activity from a single cancer cell was detected using this technique [4].

Before these papers, we have been developing the electrochemical telomerase assay (ECTA) and successfully achieved the oral cancer diagnosis using cells collected with the brushing of inside the mouth [5–7]. Principle and procedure of this method were shown in Fig. 1. As an initial stage, we utilized ferrocenyl naphthalenediimide (FND)7 as an electrochemical tetraplex DNA ligand. Other metalloccenes such as manganocene and cobaltocene are also electrochemically stable and might be useful as an electrochemically labeling probe for biological assay [8]. We chose ferrocene in our system. Ferrocene has the redox potential at the
positive side, which is not overlapped redox potential dissolved oxygen in an aqueous medium at the negative side. However, the redox potential of FND7 was a 0.45 V vs. Ag/AgCl, which region affected non-Faraday current and showed the detection limit of 50 HeLa cells [5]. When the use of FND3 instead of FND7, detection limit was improved to 10 cells [7].

According to the importance of tetraplex DNA ligand in cancer therapy, tri- or tetra-substituted naphthalene diimide have been drawing attention and tetra-substituted naphthalene diimide is known to show an improved binding affinity for tetraplex DNA [9]. Here, we designed and synthesized naphthalene diimide derivatives carrying four ferrocenyl substituents, tFND1 and tFND2 to improve the ECTA performance.

2. Experimental

2.1. Materials

FND3 and FND7 were synthesized according to the procedure previously reported [10]. The custom synthesized oligonucleotide was purchased from Genenet (Fukuoka, Japan). Oligonucleotides used in this experiment are shown in Table 1.

2.2. Synthesis of tFND1

tFND1 was synthesized as shown in Scheme 1. Brominated naphthalene tetra-anhydride ND-Br2 in 100 mL toluene was synthesized according to a previous report [11]. The mixture of 1.0 g
(2.3 mmol) of ND-Br₂ and 4.2 mL (4.0 mmol) of N-methyl-1,3-diaminopropane in a flask was irradiated with microwave (150 °C, 700 W) for 2 min. After cooling, toluene was removed under reduced pressure to obtain the blackish green viscous oil. The obtained residuum was dried at 130 °C under 4 kPa and 0.45 g (0.19 mmol) of tNDI-p-NH₂ was obtained as blue-colored solid after purified by silica gel chromatography. tFND1 was obtained as 0.20 g (0.10 mmol) of blue-colored solid after purified by Pure tFND2 identified by reversed HPLC (Fig. S4) was assigned by MALDI-TOF-MS (Fig. S2). 

### Table 1

DNA used in this experiments.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>DNA sequence</th>
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<tbody>
<tr>
<td>A-core</td>
<td>5’-AGG GTT AGG GTT AGG GTT AGGG-3’</td>
</tr>
<tr>
<td>HS-Single</td>
<td>5’-(CH₂)₆S-5’-TAG TTT TTT CTT CTT TTT TTT GTT GTT GTT GTT GTT GTT GTT-3’</td>
</tr>
<tr>
<td>HS-G4</td>
<td>5’-(CH₂)₆S-5’-TAG TTT GGG TTA GTA GGG TGA TGA GGT-3’</td>
</tr>
<tr>
<td>HS-TBTS1</td>
<td>5’-(CH₂)₆S-5’-TTT TTT TAC GTC GAC CAG AGT TAG GG-3’</td>
</tr>
</tbody>
</table>

2.3. Synthesis of tFND2

tFND2 was synthesized as shown in Scheme 2. The mixture of 0.34 g (0.80 mmol) of ND-Br₂ and 1.5 g (4.8 mmol) of p-NH₂ (Boc) [10] in a flask was refluxed for 71 h. After cooling, toluene was removed under reduced pressure, and 1 L of Milli-Q water was added and carried out ultrasonic irradiation. After filtration and removed to water under reduced pressure, 0.84 g (0.59 mmol) of purple-colored viscous solid was obtained. This solid mainly containing tNDI-p-Boc was added in 10 mL of trifluoroacetic acid and stirred for 90 min at room temperature. Solvent was removed under reduced pressure and 0.45 g (0.19 mmol) of tNDI-p-NH₂ was purified as blue-colored content at 13 min by the medium pressure preparative liquid chromatography under gradient conditions, where the acetonitrile content in 0.1% trifluoroacetic acid was changed linearly from 5 to 70% over 40 min at a flow rate of 20 mL/min with detection at 254 nm.

Five mL of DMF containing 0.30 g (0.13 mmol) of tNDI-p-NH₂ and 5 mL of trimethylamine was added with the mixture of 20 g (0.76 mmol) of ferrocenylpropanoic acid [10] in 5 mL of DMF and 5 mL of trimethylamine and consequently added with 2.4 g (4.5 mmol) of 1H-Benzotriazol-1-yl-oxy-tri(pyridylidino)phosphonium hexafluorophosphate (PyBOP, Watanabe Chemical Industries LTD, Hiroshima, Japan) and 0.61 g (4.53 mmol) of 1-Hydroxy-1H-benzotriazole hydrate (Watanabe Chemical Industries LTD) and stirred for 27 h at room temperature in shaded flasks. After removed the solvent and added 80 mL of chloroform and filtrated. The filtrate was washed with 30 mL of saturated sodium bicarbonate aqueous solution three times, and this filtrate was dried over anhydride Na₂SO₄. After added with diethyl ether and carried out ultrasonic irradiation. After filtration, precipitates were subjected to silica gel chromatography using the mixture of CHCl₃: methanol (8:2) as eluent. tFND2 was obtained as 0.20 g (0.10 mmol) of blue-colored solid after purified by Pure tFND2 identified by reversed HPLC (Fig. S4) was assigned by MALDI-TOF-MS (Fig. S5).

### Scheme 2

Synthetic route of tFND1.
irradiation of O2 plasma at 0.5 Torr for 30 s (Cute-MP, Femto Science, Gyeonggi, South Korea) and dipped into 4 μL of 1.0 mM 6-mercaptohexanol and kept for 1 h at 45°C. After washed with Milli-Q water and blow off by nitrogen gas. For HS-Single and HS-G4 (Table 1) immobilized electrode, printed gold electrode (φ 1.6 mm, Tanaka Kikinzoku Kogyo) was irradiation of O2 plasma at 0.5 Torr for 30 s and dipped into 10 μL probe DNA in 100 mM NaCl and kept for 2 h at 37°C and was subsequently dipped 4 μL of 1 mM 6-mercaptohexanol and kept for 1 h at 45°C. After washed with Milli-Q water and blow off by nitrogen gas.

2.5.2. Electrochemical measurements

CV measurements were using with three conﬁguration of reference electrode of Ag/AgCl, Pt counter electrode, and printed gold electrode as working electrode using Electrochemical Analyzer Model 650C (CH Instrument, Austin, TX) with the electrolyte of 100 mM AcOH-AcOK buffer (pH 5.5) and 100 mM KCl containing 50 μM tFND1 or 100 mM AcOH-AcOK buffer (pH 5.5), 100 mM KCl, and 40% DMSO containing 20 μM tFND2. Chronocoulometry (CC) was carried out according to the method previously reported [13]. SWV was measured in 100 mM AcOH-AcOK buffer (pH 5.5), 100 mM KCl, and 5 μM tFND1 or 100 mM AcOH-AcOK buffer (pH 5.5), 100 mM KCl, and 40% DMSO containing 5 μM tFND2.

2.5.3. Electrochemical telomerase assay (ECTA)

HS-T8TS1 (Table 1) was immobilized on the printed electrode Fig. S8, and the elongation reaction on this electrode was carried out according to the procedure previously described [7]. The electrode was washed with dipping into 10 mL of MilliQ water and RNase free water (0.2 U/μL RNase inhibitor) before the telomerase reaction. The telomerase reaction was then run in 10 μL of 50 mM Tris-HCl (pH 8.0) containing 0.01–50 cells/μL Hela cell lysate, 1.0 mM MgCl2, 50 mM KCl, 0.10 mM 2-mercaptoethanol, 0.10 mM spermidine, 10 μM dNTP mixture at 37°C for 30 min. The electrode was washed with dipping into 10 mL of MilliQ water, 1× PBS, and 100 mM AcOH-AcOK buffer (pH 5.5) containing 100 mM KCl before SWV measurement (Fig. S8). Before and after the telomerase reaction, SWV measurements were carried out in 100 mM AcOH-AcOK buffer (pH 5.5), 100 mM KCl, and 40% DMSO containing 20 μM tFND2. Telomerase activity was evaluated using the following the equation: \[ \Delta i = \frac{(i-i_0)}{i_0} \times 100 \], where \( i \) and \( i_0 \) are referred to current before and after telomerase reaction, respectively.

3. Results and discussion

3.1. Electrochemical behavior of tFDN1 and tFND2

Fig. 2 shows cyclic voltammogram of 50 μM tFDN1 was measured in a 6-mercaptohexanol-immobilized electrode in 100 mM AcOK-AcOH buffer (pH 5.5) and 100 mM KCl to obtain the following parameters: 432 mV of redox potential (\( E_{1/2} \)) from 453 mV of oxidation potential (\( E_{pa} \)), 411 mV of reduction potential. From low servility of tFND2 in an aqueous medium, tFND2 was dissolved in DMSO as stocked solution and the cyclic voltammogram of 20 μM tFND2 was measured in the 6-mercaptohexanol-immobilized electrode under 100 mM AcOK-AcOH buffer (pH 5.5), 100 mM KCl, and 40% DMSO to obtain the following parameters: 261 mV of the redox potential (\( E_{1/2} \)) from 292 mV of the oxidation potential (\( E_{pa} \)), 230 mV of the reduction potential. Since FND3 carrying same ferrocenyl substituents has a 190 mV of \( E_{1/2} \) [11], tFND2 is positively shifted to 70 mV and this might come from the effect of the liquid junction potential in an aqueous electrolyte containing DMSO [14].

Even after the consideration of this factor, the redox potential of tFND2 shows more negatively than that of tFDN1. Scan rate dependence of the peak current of tFDN1 and tFND2 in CV were measured and these currents were correlated with \( v^{1/2} \), which is suggesting their electron transfer from the electrode was diffusion process.

3.2. Interaction of tFDN1 and tFND2 with tetraplex DNA

A-core is a part of the human telomere DNA sequence and is known to have a mixture of anti-parallel and hybrid tetraplex structures [15]. CD spectra of A-core upon addition of tFND1 and...
Fig. 2. Cyclic voltammogram of tFND1 (A) and tFND2 (B). Measurement was carried out using 6-mercaptohexanol-immobilized electrode in 50 μM tFND1, 100 mM AcOH-AcOK buffer (pH 5.5), and 100 mM KCl (A) or 20 μM tFND2, 100 mM AcOH-AcOK buffer (pH 5.5), 100 mM KCl, and 40% DMSO.

Fig. 3. CD spectra of 1.5 μM a-core upon addition of tFND1 (A) and tFND2 (B) in 100 mM AcOH-AcOK buffer (pH 5.5) and 100 mM KCl. Job's plots in the case of tFND1 (C) and tFND2 (D). CD change at 290 nm of A-core was plotted against molar ratio of [tFND]/[A-core].
tFND2 in 100 mM AcOH-AcOK buffer (pH 5.5) and 100 mM KCl. Fig. 3 (A) shows the positive Cotton effect at 290 nm and the negative one at 240 nm with the positive shoulder around 270 nm. Since this shoulder signed of the hybrid structure was increased with an increasing the amount of tFND1, tFND1 seems to induce the hybrid structure upon the binding to A-core.

On the other hand, the intensity of the CD bands increased upon the addition of tFND2 up to twice of A-core concentration, suggesting binding of tFND2 with tetraplex DNA both tetraplex structure (Fig. 3B). Job’s plots were conducted using the CD intensity change at 290 nm, and the binding ratio of tFND1 and tFND2 to A-core was one and two as shown in Fig. 3C and D.

3.3. Estimation of selectivity between tetraplex DNA and single stranded DNA on the electrode

Under the ECTA, the electrochemical ligand is required the higher current signal for tetraplex DNA over single stranded one resulting in higher current signal. Current ratio (G4/Single) for tetraplex DNA to single stranded DNA on the electrode was evaluated from the current measurements of several FND derivatives in the tetraplex DNA or mercaptoethanol DNA immobilized electrode. Firstly, the amount of the tetraplex (HS-G4, 24 mer) or the single stranded DNA (HS-Single, 24-mer) immobilized on the electrode was estimated by CC method with the procedure previously described [12]. Both DNAs was immobilized with 4.6 × 10¹¹ molecules/cm² and 8.6 × 10¹² molecules/cm², respectively. Peak currents of these electrodes in tFND2 were 5.9 × 10⁻⁸ A and 8.3 × 10⁻⁸ A, respectively and the division of peak current by the DNA-immobilized density gave the concentration amount of tFND2 normalized by DNA strand immobilized on the individual electrode. Current ratio (G4/Single) was calculated by the division of this normalized value in the case of tetraplex DNA by that in the case of single stranded DNA. According to this calculation, the current ratio (G4/Single) was 1.1, 1.6 and 1.8 for FND7 FND3, and tFND1, respectively, whereas Current ratio (G4/Single) for tFND2 was 13.5 suggesting the tFND2 has the highest preference for tetraplex DNA over single stranded DNA and has potentiality to improve ECTA performance (Fig. 4). tFND2 has piperazine part in the linker. It is reported that 4-substituted naphthalenediimide strongly interacts with the phosphate of quadruplex DNA by introduced piperazine moieties into the linker portion [16]. The electrochemical measurement result was in agreement with the previous reports [16,17].

3.4. Detection of telomerase activity in ECTA using tFND2

Telomerase activity was estimated by ECTA using tFND1 or tFND2. In the case of tFND1 (Fig. S9), Δi was 45 ± 20% after treatment of 0.1 cells/µL HeLa cell. However, too small i₀ values and standard deviation, in this case, was too large to quantify the telomerase activity. On the other hand, in the case of tFND2, the i₀ value of 0.28 µA increased to the i value of 0.76 µA after treated with 50 cells/µL HeLa cells (Fig. 5A). Concentration dependence of Δi value in HeLa cell is shown in Fig. 5B and shows the detection limit of 0.1 cells/µL using the 3σ method. The current increase was diminished about 80% compared with the case of 50 cells/µL HeLa cell treatment for heating treatment of this sample at 90 °C. This suggested that the obtained signal come from telomerase activity.

4. Conclusion

Newly synthesized tFND1 and tFND2 bound to tetraplex DNA with 1:1 or 1:2 binding ratio, respectively. Preference of tFND2
between tetraplex DNA and single stranded one was higher than that of tFND1, and the stable current signal was observed in the ECTA system using tFND2 with quantitative change around 0.1–50 cells/μL and detection limit of 0.1 cells/μL.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jorganchem.2019.06.028.

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