A Comparison of Electrochemical DNA Probes Possessing an Isomeric Ferrocene-Diamidopyridine Conjugate for SNPs Detection on Au(111)*

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Electrochemical single nucleotide polymorphisms (SNPs) detection was investigated on gold surface modified with 16-base-pair duplex DNAs. In these duplexes, probe DNAs consisted of complementary sequences for target DNAs and a series of ferrocene-diamidopyridine conjugates as an electrochemically active moiety. Regardless of isomeric structures in the conjugates, all the DNA probes showed electrochemical discrimination between fully matched DNAs and SNPs. A detailed comparison was carried out for these probes. [DOI: 10.1380/ejssnt.2005.393]

Keywords: Electrochemical methods; Gold; Single nucleotide polymorphism; Ferrocene

I. INTRODUCTION

Rapid and sensitive detection of biological targets on chemically modified surfaces has been widely investigated for developing high-throughput sensors [1–4]. Especially, DNA-based sensors have a potential for the application to large-scale typing of single nucleotide polymorphisms (SNPs) [5, 6]. SNPs are common sequence variations among DNAs of individuals and relate to genetic basis disease and drug response (pharmacogenomics) [7, 8]. Therefore, a simple and inexpensive protocol for large-scale SNPs assay of individuals has been desired in post-synthetic disease and drug response (pharmacogenomics) [7, 8]. SNPs are common sequence variations

structures. Here we show the electrochemical SNPs detection on Au(111) electrodes modified with new DNA probes, HS-DNA-Fc(ϕR) and HS-DNA-Fc(Achiral) that have isomeric structures in the nucleoside residue (Fig. 1).

II. EXPERIMENTAL

Materials. The (ϕR)-phosphoramidite 1 ([α]25\textdegree +108.4 (c 1.00, CHCl3)) of the nucleoside analogue for solid-phase DNA synthesis was easily prepared from known (ϕR)-2-iodoferroacenecarboxaldehyde [13] in the same manner to that described for the corresponding (ϕS)-phosphoramidite 2 ([α]25\textdegree -108.1 (c 1.04, CHCl3)) (Scheme 1 (Fig. 2)) [12]. The synthetic procedure of the achiral phosphoramidite 3 is shown in Scheme 2 (Fig. 3). Synthesis of DNA probes, preparation of duplex-modified electrodes, and electrochemical measurements were performed as previously reported [12]. The starting materials, 1'-iodoferroacenecarboxaldehyde (4) [14] and 2,6-diacetamido-4-ethylpyridine [14] were prepared according to literature procedures. Other materials were all commercially available.

General. 1H and 13C NMR spectra were recorded on a Varian Gemini 300 spectrometer (300 MHz for 1H and 75 MHz for 13C). IR spectra were recorded on a JASCO-FT/IR 460 plus spectrometer. ESI-HRMS and ESI-HRMS analyses were carried out on a JEOL-JMD-D300 and a JEOL-JMS-T100LC mass spectrometer, respectively. Melting points were determined with Yanako-MP-500D and not corrected. All synthetic reactions were carried out under argon atmosphere. THF was freshly distilled from sodium benzophenone ketyl before use, and other solvents were purified with standard methods [15].

1-Ethyl-1'-iodoferrocene (5). To a THF (40 mL) suspension of methyl(triphenyl)phosphonium bromide (8.4 g, 23.5 mmol) was added a THF (20 mL) solution of t-BuOK (2.6 g, 23.5 mmol) at room temperature, and the reaction mixture was stirred for 30 min at that

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temperature. To the mixture was added a THF (20 mL) solution of 4 [14] (3.2 g, 9.4 mmol) at room temperature. After stirring for an additional 1 h, the reaction mixture was quenched by addition of H$_2$O (10 mL), diluted with AcOEt (100 mL), and washed with brine. The organic layer was evaporated and chromatographed (silica gel; eluent: hexane = 1 : 9) to give 5 as an oil: yield 94% (3.0 g); IR (neat) 3084, 1630 cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$ 6.45 (dd, $J$ = 17.4, 10.8 Hz, 1 H), 5.40 (dd, $J$ = 17.4, 1.4 Hz, 1 H), 5.13 (dd, $J$ = 10.8, 1.4 Hz, 1 H), 4.28$\sim$4.31 (m, 4 H), 4.23 (dd, $J$ = 2.0, 1.8 Hz, 2 H), 4.07 (dd, $J$ = 1.8, 1.8 Hz, 2 H), 3.68$\sim$3.78 (m, 2 H), 2.62 (t, $J$ = 6.6 Hz, 2 H), 1.65 (t, $J$ = 15.3 Hz, 1 H); $^{13}$C NMR (CDCl$_3$) $\delta$ 133.14, 112.19, 85.04, 75.58, 71.58, 69.94, 69.32, 40.32; HRMS (ESI) calcd for MNa$^+$, C$_{12}$H$_{13}$OFeINa: 378.9258; found 378.9246.

1-(2-Hydroxyethyl)-1'-iodoferrocene (6). To a THF (90 mL) solution of 9-BBN (3.2 g, 13.3 mmol) was added a THF (60 mL) solution of 5 (3.0 g, 8.9 mmol) dropwise at room temperature over 10 min period. The reaction mixture was stirred at reflux for 1 h. After cooling to room temperature, the mixture was added EtOH (25 mL), 2$N$ NaOH (25 mL), and a 30% H$_2$O$_2$ aqueous solution (20 mL) successively at room temperature. The reaction mixture was stirred at 50°C for 1 h, diluted with AcOEt (100 mL), and washed with brine. The organic layer was evaporated and chromatographed (silica gel; eluent: hexane = 1 : 9) to give 6 as an oil: yield 86% (2.7 g); IR (neat) 3345, 2925 cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$ 4.33 (dd, $J$ = 2.0, 2.0 Hz, 2 H), 4.11$\sim$4.14 (m, 4 H), 4.07 (dd, $J$ = 1.8, 1.8 Hz, 2 H), 3.68$\sim$3.78 (m, 2 H), 2.62 (t, $J$ = 6.6 Hz, 2 H), 1.65 (t, $J$ = 15.3 Hz, 1 H); $^{13}$C NMR (CDCl$_3$) $\delta$ 86.32, 74.99, 71.50, 69.28, 63.25, 40.48, 32.12; HRMS (ESI) calcd for MNa$^+$, C$_{12}$H$_{13}$OFeINa: 378.9258; found 378.9246.

2,6-Diacetamido-4-[2-(1'-hydroxyethyl)ferrocenyl]ethynyl]pyridine (7). A DMF$-i$Pr$_2$NH (8$\sim$10 mL) mixed solution of 6 (164 mg, 0.64 mmol), 2,6-diacetamido-4-ethynylpyridine [14] (150 mg, 0.69 mmol), (Ph$_3$P)$_4$Pd (58 mg, 0.05 mmol), and Cu(OAc)$_2$·H$_2$O (10 mg, 0.05 mmol) was stirred at 100°C for 2 h. After removal of the solvent, the residue was poured into water and extracted with CHCl$_3$. The CHCl$_3$ extract was evaporated and chromatographed (silica gel; eluent: CH$_2$Cl$_2$ : MeOH = 19 : 1) to give 7 as an orange solid: yield 99% (226 mg);Mp 185-190°C.

FIG. 1: Chemical structures of probe DNAs and illustration for electrochemical SNPs discrimination with a ferrocene-modified, wire-like DNA probe.
FIG. 2: Scheme 1.

Scheme 1.

\[
\begin{align*}
\text{(R)-1,2,4-butane diol} & \quad \text{CHO} \\
\text{Fe} & \quad \text{CHO} \\
\text{(S)-1,2,4-butane diol} & \quad \text{CHO}
\end{align*}
\]

\[\text{(pR)-phosphoramidite 1}\]

\[\text{(pS)-phosphoramidite 2}\]

FIG. 3: Scheme 2.

Scheme 2.\textsuperscript{a}

\[\begin{align*}
4 & \quad \text{CHO} \\
5 & \quad \text{CH}=\text{CH}_2 \\
6 & \quad \text{CH}_2\text{CH}_2\text{OH} \\
7 & \quad \text{achiral phosphoramidite 3}
\end{align*}\]

\textsuperscript{a}Key: (a) Ph\textsubscript{3}P\textsubscript{2}CH\textsubscript{3}Br, t-BuOK, THF; (b) i) 9-BBN, THF; (ii) 2N NaOH, EtOH; (iii) 30% H\textsubscript{2}O\textsubscript{2}; (c) 2,6-
diacetamido-4-ethylpyridine, (Ph\textsubscript{3}P\textsubscript{2}Pd, Cu(OAc)\textsubscript{2}H\textsubscript{2}O, i-Pr\textsubscript{2}NH, DMF; (d) NC(CH\textsubscript{2})\textsubscript{2}OPClN(Pr-i\textsubscript{2}), 4-(dimethylamino)pyridine, i-Pr\textsubscript{2}NET, CH\textsubscript{2}Cl\textsubscript{2}.

Phosphoramidite 3. To a CH\textsubscript{2}Cl\textsubscript{2} (10 mL) solution of 7 (45 mg, 0.10 mmol) were added a CH\textsubscript{2}Cl\textsubscript{2} (4 mL) solution of 4-(dimethylamino)pyridine (6 mg, 0.050 mmol) and i-Pr\textsubscript{2}NH (0.12 mL, 0.70 mmol) at 0\textdegree C. After the reaction mixture had been stirred for 5 min, to the mixture was added NC(CH\textsubscript{2})\textsubscript{2}OPClN(Pr-i\textsubscript{2}) (0.07 mL, 0.30 mmol) at that temperature. After warming to room temperature with stirring over 2 h period, the mixture was diluted with CH\textsubscript{2}Cl\textsubscript{2} (40 mL) and washed with a saturated NaHCO\textsubscript{3} aqueous solution. The organic layer was evaporated and purified by HPLC (ODS; eluent, MeOH) to give 3 as an orange oil (a mixture of diastereomers): yield 54\% (35 mg); IR (neat) 3290, 2965, 2209, 1681, 1613, 1552, 1414 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \(\delta\) 7.94 (br s, 2 H), 7.68 (s, 2 H), 4.47~4.81 (m, 2 H), 4.25~4.26 (m, 2 H), 4.17~4.19 (m, 2 H), 3.54~3.56 (m, 4 H), 3.52~3.54 (m, 2 H), 2.58 (t, \(J = 6.5\) Hz, 2 H), 2.20 (s, 6 H), 1.14 (t, \(J = 7.2\) Hz, 12 H); \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \(\delta\) 168.14, 149.16, 136.46, 117.58, 110.80, 93.97, 86.22, 84.51, 72.26, 70.32, 69.94, 69.49, 69.43, 63.99, 63.93, 63.78, 58.36, 58.12, 43.01, 42.84, 30.55, 30.45, 24.76, 24.63, 24.53, 24.46, 20.35, 20.27; HRMS (ESI) calcd for MN\textsubscript{a}\textsuperscript{+}, C\textsubscript{32}H\textsubscript{40}N\textsubscript{5}O\textsubscript{4}PFeNa: 668.2066; found 668.2036.

MALDI-TOF mass measurements. MALDI-TOF mass spectra were recorded on a Bruker-Daltonics-Autoflex mass spectrometer with 3-hydroxypicolinic acid as a matrix. The peak of ionized molecule was found for DNA-Fc(pR) (exact mass for MH\textsuperscript{+}: 5083.88), HS-DNA-Fc(pR) (exact mass for MH\textsuperscript{+}: 5237.86), HS-DNA-Fc(Achiral) (exact mass for MH\textsuperscript{+}: 5237.86) and HS-DNA\textsubscript{2}-Fc(pR) (exact mass for MH\textsuperscript{+}: 5220.83) at 5086.96, 5238.00, 5237.09, and 5221.00, respectively.
III. RESULTS AND DISCUSSION

In our previous report, electrochemical SNPs detection was performed with the probe DNAs possessing a ferrocene moiety of pS planer chirality [12]. This chirality happens at a stage of synthesis for the nucleoside analogue and is originated from the configuration of a chiral reactant, 1,2,4-butanetriol (Scheme 1 (Fig. 2)). When a racemic nucleoside analogue was used, starting from commercially available racemate of 1,2,4-butanetriol, the corresponding probe DNA was synthesized as a mixture of diastereomers. Reverse-phase HPLC purification could separate these two DNA probes bearing each chirality of pS or pR in the nucleoside residue. Although a detailed study was carried out for the probe DNA containing the pS chirality [12], the corresponding probe of the pR one remains to be investigated. To clarify the influence of isomeric structures in the ferrocene unit on electrochemical responses, we examined the DNA probes possessing the pR ferrocene moiety. Furthermore, an achiral version of the ferrocene unit was also synthesized (Scheme 2 (Fig. 3)) and derivatized to the corresponding probe. Both the new probes were identified by MALDI-TOF mass measurements.

To unambiguously compare, the sequence of a 16-mer DNA probe (3'-HS(CH₂)₃-AGT ACA GTC ATC GCG Fe-5') is selected on the basis of our previous report [12]. In this sequence, the residue of the ferrocene-modified nucleoside analogue is abbreviated to Fe, and probes are referred to as HS-DNA-Fc(pS), HS-DNA-Fc(pR), and HS-DNA-Fc(Achiral) corresponding to the chirality in each of the ferrocene moiety (Fig. 1). The sequences of a fully matched complement 8 and its SNP 9 examined in this study are 5′-TCA TGT CAG TAG CGC T-3′ and 5′-TCA TGT CA'G TAG CGC T-3′ (underlined residue is a mismatched base), respectively.

In advance, UV and circular dichroism (CD) spectra for duplexes between the probe and the complements were measured in aqueous media. Therefore, a thiol-free version of HS-DNA-Fc(pR), DNA-Fc(pR) (3′-AGT ACA GTC ATC GCG Fe(pR)-5′) was synthesized and investigated for the duplex formation. From the UV-melting profiles, the \( T_m \) values of 62.4 and 47.5°C were determined for the duplexes from the fully matched complement 8 and the SNP 9, respectively (Fig. 4). As expected, the duplex from 9 showed the lower \( T_m \) value compared with that of the duplex from 8 because of the presence of a mismatched base pair in the middle of the sequences. However, even with 9, DNA-Fc(pR) firmly forms the duplex structure at 25°C, which is the temperature for the measurements in electrochemical SNPs detection. The duplex formation was also confirmed on the basis of CD in 1 M NaClO₄ at 25°C. Cotton effects in the wavelength region of 300 to 200 nm reflect the secondary structures of double-stranded DNAs. A mixture of DNA-Fc(pR) and the SNP 9 showed an almost identical CD spectrum in that region with those not only of DNA-Fc(pR) 8 but also of DNA-Fc(pS) 9 [12]; characteristic of a typical double stranded B-form DNA [16] (Fig. 5).

Square wave voltammetry (SWV) measurements were performed at the electrodes modified with duplexes from the new DNA probes scanning from 0 to +0.6 V vs. Ag/AgCl in 1 M NaClO₄. When applying the duplex from HS-DNA-Fc(pR) and the fully matched complement 8, an intense anodic peak appeared at +0.30 V, resulted from the oxidation of the ferrocene moiety (Fig. 6A). This potential is almost the same value in the combination of HS-DNA-Fc(pS) and 8 [12]. On the other hand, no obvious SWV response was observed from the duplex with the mismatched complement 9. In the same manner, we also applied the duplexes from HS-DNA-Fc(Achiral) with 8 and 9 for the electrochemical measurements (Fig. 6B). A quite similar discrimination at +0.30 V was seen for these duplexes as in the cases for HS-DNA-Fc(pR) and HS-DNA-Fc(pS). Regardless of the isomeric struc-

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FIG. 4: UV-melting curves at 260 nm of DNA-Fc(pR) 8 (solid line) and DNA-Fc(pR) 9 (dotted line). Absorbance vs. temperature profiles of the duplexes were measured by using a JASCO-V-560 spectrophotometer with a peltier and a temperature controller in a range from 20 to 90°C. The solution for the measurement contained DNA-Fc(pR) (1 \( \mu \)M) and 1 equiv of 8 or 9 (1 \( \mu \)M) in 1 M NaClO₄.

FIG. 5: CD spectra at the duplex concentration of 1 \( \mu \)M for DNA-Fc(pR)-8 (red, solid line), DNA-Fc(pR)-9 (blue, solid line), DNA-Fc(pS)-8 (red, dotted line), and DNA-Fc(pS)-9 (blue, dotted line) in 1 M NaClO₄ at 25°C. CD spectra were recorded on a JASCO-J-720WI spectropolarimeter.
FIG. 6: Uncorrected SWV profiles at the gold working electrodes modified with (A) HS-DNA-Fc(pR)·8 (fully matched duplex, solid line) and HS-DNA-Fc(pR)·9 (mismatched duplex, dotted line), and (B) HS-DNA-Fc(Achiral)·8 (fully matched duplex, solid line) and HS-DNA-Fc(Achiral)·9 (mismatched duplex, dotted line).

FIG. 7: Uncorrected SWV profiles at the gold working electrodes modified with the duplexes from HS-DNA2-Fc(pR) with fully matched DNA10 (red line), 248A SNP (blue line), 249 SNP (light blue line), and 248T SNP (green line). Sequences are 5'-TGA ACC GGA GGC CCA T-3' for fully matched DNA10, 5'-TGA ACC AAGA GGC CCA T-3' for 248A SNP, 5'-TGA ACC AGTC CCA T-3' for 249 SNP, and 5'-TGA ACT GGA GGC CCA T-3' for 248T SNP (underlined residues are the mismatched bases).

IV. CONCLUSIONS

We demonstrated electrochemical SNPs discrimination on Au(111) modified with DNA probes possessing a series of isomeric structures in a ferrocene unit. Regardless of stereochemistry in the ferrocene moiety, all the DNA probes performed similar electrochemical responses for SNPs discrimination. This tolerance of the ferrocene unit for detecting SNPs is an important additional advantage for our "wire-like" DNA probes. Development of more simple and easy-made ferrocene units is now underway.

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