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Synthesis of ferrocenylcarbodiimide as a convenient electrochemically active labeling reagent for nucleic acids

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Abstract—Ferrocenylcarbodiimides carrying different redox potentials, **1** and **2**, were designed and synthesized as convenient electrochemically active labeling reagents for nucleic acids, which may be used as dually labeling reagents of nucleic acids like Cy3 and Cy5 dyes. These reagents could react with the imino unit of thymine or guanine base on DNA or of uracil base on RNA under a basic buffer condition to yield a labeled product quantitatively in a short period of time. The current responses of the labeled DNAs in square wave voltammetric (SWV) measurement showed a good linear correlation with the amount of the hybridized ones. DNAs labeled with the two different reagents, **1** and **2**, could be detected electrochemically at different potentials after hybridization with a DNA probe-immobilized gold electrode.

1. Introduction

Recently, conventional DNA detecting systems are required in the gene diagnosis and many DNA sensing methods based on electrochemical techniques have been reported,[1–19] as they are expected to realize a convenient gene testing system, which enables direct electronic readout and miniaturization with good cost performance and high sensitivity. Furthermore, they are expected as one of the solutions for the carrier-type gene testing chip and can be applied to various purposes of gene testing such as the point-care test.[20] A variety of electrochemical DNA detecting techniques have been reported by using electrochemically active DNA ligands such as intercalating molecules,[4,7–8,21] or DNA labeling with electrochemically active reagents.[2,5,14–15,19,24–28] Direct electrochemical DNA detection has been achieved under limited conditions using special electrodes and electrolytes.[1,22–23] Ferrocene is often used as an electrochemically active reagent,[2,5,7–9,11,14–15,19,21,24–29] as its reversible redox potential appears where dissolved and atmospheric oxygen does not interfere with the measurement. Oligonucleotides labeled with ferrocene have been used as a DNA probe in electrochemical DNA sensing. Introduction of a ferrocence moiety to oligonucleotides was achieved by a ferrocenyl amide reagent for automated DNA synthesis,[5,19,25–27,30–34] ferrocenyl nucleotide triphosphate as substrate of DNA polymerase,[9,35–38] and the reaction of an activated ester of ferrocene with the amino linked oligonucleotides.[2,11,14,39–41] Direct modification of DNA with ferrocene was also reported by using the Sonogashira reaction of ferrocenyl-propargylamide with halogenated nucleic base of DNA.[42,43] However, all of these methods described here, suffer time-consuming steps and therefore, simpler and more effecient ferrocenylation methods for DNA need to be devised.

To achieve a simple labeling method for nucleic acids, ferrocenylcarbodiimide derivatives, **1** and **2**, carrying different redox potentials were designed and synthesized. Water-soluble carbodiimide derivatives are known to react with the imino moiety of thymine and guanine bases on DNA or of uracil base on RNA reversibly under basic conditions[44–47] with excellent yield and therefore, ferrocenylcarbodiimide derivatives should react with DNA and RNA in the same manner, thereby rapidly labeling natural single stranded DNA or RNA fragments with ferrocene as depicted in Scheme 1. Since the redox potential of ferrocene can be altered readily by changing the nature of its substituent, ferrocenylcarbodiimide derivatives having different redox potentials may be prepared by designing a linker connecting ferrocene with carbodiimide parts. Once prepared, such compounds will serve as an important tool to enable competitive analysis of two different samples labeled differentially with the ferrocenyl groups with a different redox potential.

Keywords: Ferrocenylcarbodiimide; Electrochemical detection; DNA; RNA; Dual labeling; Competitive hybridization.
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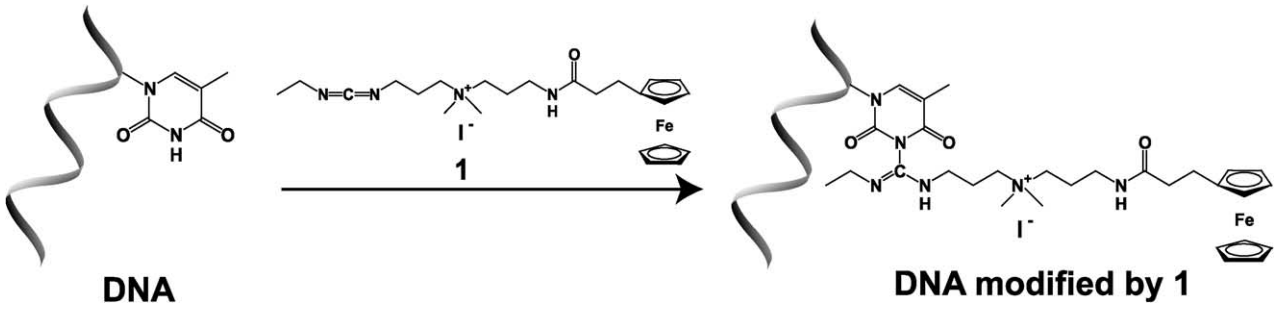


Scheme 1. Example of a ferrocene-modification reaction for thymine base of nucleic acid with **1**.

The principle of this method is illustrated in Figure 1, which is similar to the expression analysis based on a DNA microarray coupled with a dual labeling of standard and sample DNAs with Cy3 and Cy5 dyes, respectively. We assessed the feasibility of electrochemical gene expression analysis by using ferrocenyl oligonucleotides, which are labeled by the activated ester of ferrocenecarboxylic acid and ferrocenepropionic acid.[48] Other electrochemical gene expression analyses were reported by using 7-deaza guanine and adenine bases incorporated by PCR[49] and nucleoside triphosphate derivatives carrying ferrocene and anthraquinone units.[50] These reports underscored the potential importance of the electrochemical gene expression analysis and usefulness of ferrocenylation reagents having different redox potentials.

In this paper, the synthetic methods of **1** and **2** were established by surveying an effective condition for the reaction with single stranded DNA or RNA. The stability of the ferrocenyl oligonucleotides thus obtained was also evaluated by the melting curve analysis. Finally, we succeeded in the electrochemical detection of target DNA by using **1** and **2**, to suggest that the electrochemical gene expression analysis based on DNA or RNA labeled with **1** or **2** is promising for practical use.

2. Results and discussion

2.1. Synthesis of ferrocenylcarbodiimides and their stability in aqueous solution

Ferrocenylcarbodiimides **1** and **2** were synthesized as shown in Scheme 2. The carbodiimide function was generated by the method described previously.[51] Integrity of **1** and **2** was assessed by ^{1}H NMR and FT IR measurements, in which characteristic IR absorption at 2129 cm^{-1} shown in Figure 2 due to the carbodiimide group was strong evidence for the structure. Cyclic voltammograms determined in 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$ revealed a one-electron redox reaction with $E_{1/2}=$ 207 mV and ΔE peak $=63$ mV for **1** and $E_{1/2}=443$ mV and $\Delta E=71$ mV for **2** (Fig. 3). These half-wave currents were shifted toward the positive potential side from those of ferrocenylpropionic acid ($E_{1/2}=171$ mV) and ferrocenecarboxylic acid ($E_{1/2}=328$ mV), due presumably to a difference in their cationic and anionic characters. The stability of carbodiimides **1** and **2** was tested in several kinds of buffer by monitoring a change in the intensity of the absorption at 2130 cm^{-1} in FT IR. The carbodiimide function was destroyed within 4 or 12 h in acetic acid buffer (pH 5.6) or phosphate buffer (pH 7.0), respectively (data not

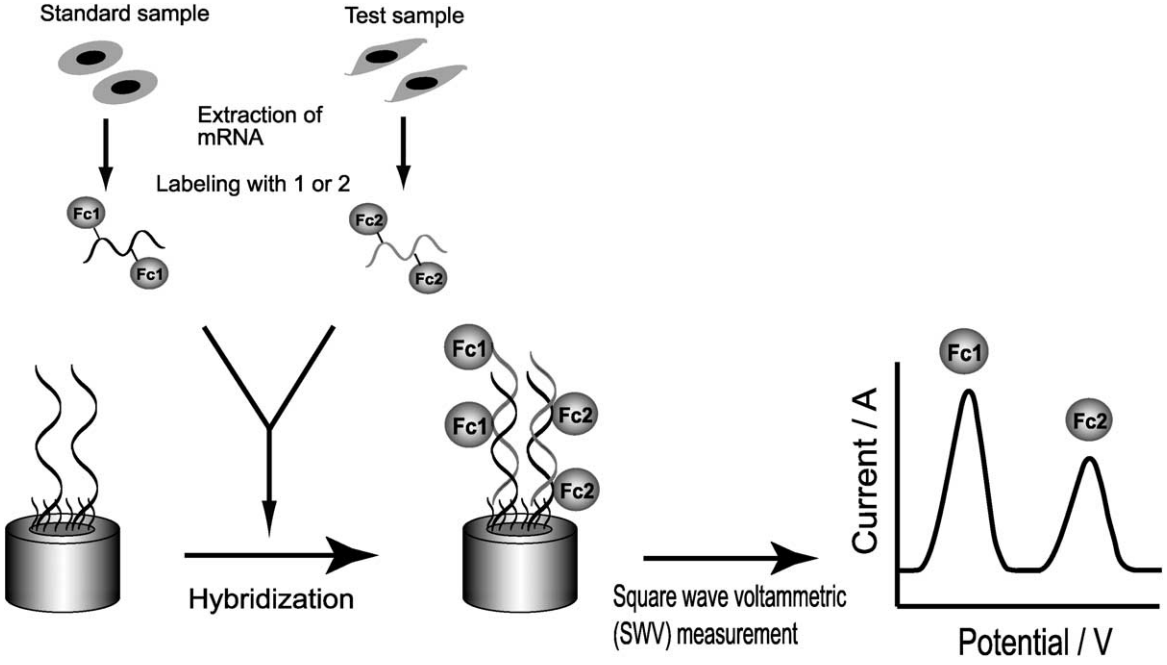
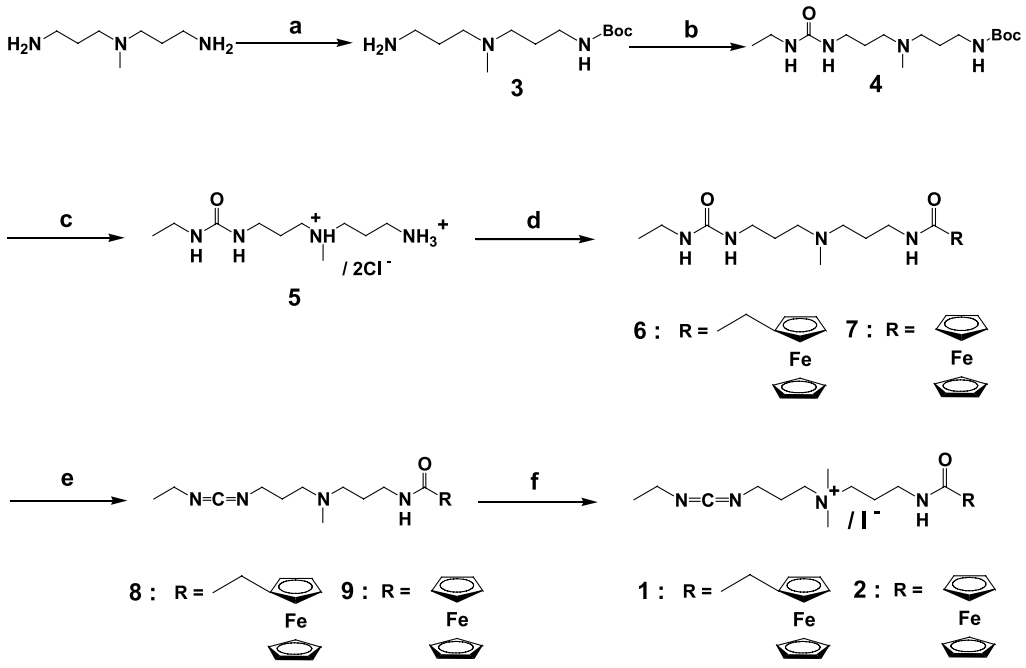


Figure 1. Principle of the electrochemical differential hybridization assay.



Scheme 2. Synthetic route to **1** and **2**. The reaction conditions are as follows: (a) *S-tert*-butoxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine/1,4-dioxane; (b) ethylisocyanate/dry ether; (c) HCl/1,4-dioxane; (d) ferrocenepropionic acid or ferrocenecarboxylic acid/PyBOP/HOBt/TEA/$CHCl_3$; (e) TsCl/TEA/dryCH_2Cl_2, reflux; (f) CH_3I/dry ether.

shown). However, it was stable over 1 day in borate buffer (pH 8.5, 9.0 and 9.5). These data are in good agreement with the results on carbodiimide derivatives described previously.[47]

2.2. Reactivity for DNA

The reactivity of **1** and **2** was studied with several kinds of DNAs, namely **D1** and **D2** carrying one thymine or guanine base at the 5′-terminus of a decamer, respectively, **D3** carrying one thymine base in the middle of a decamer, and **D4** carrying a thymine base at the terminus of a 20-mer (Table 1). The reactivity was assessed from the peak intensities of the modified and unmodified DNA in HPLC. Typical HPLC traces before and after reaction of **1** with **D1** at pH 8.5 for 12 h are shown in Figure 4. The peak at 23 min of retention time was a 1:1 adduct of **1** with **D1** as deduced by MALDI TOF MS measurement shown in Figure 5. Products of **D2**–**D4** with **1** or **2** could also be assigned as 1:1 adduct analogously (data not shown). No HPLC change was observed upon reaction of **1** or **2** with **D9** as negative control (Table 1). These results demonstrated that **1** and **2** could react with thymine and guanine bases on DNA.

In the next step, the reactivity of **1** and **2** with DNA was studied at different pH. The time course of the reaction of **1** with **D1** or **D9** at pH 8.5, 9.0 or 9.5 is shown in Figure 6A. Quantitative reaction occurred with **D1** carrying a thymine moiety and **1** within 10 h, whereas no reaction was observed with **D9**. Since the imino moiety of thymine or guanine base

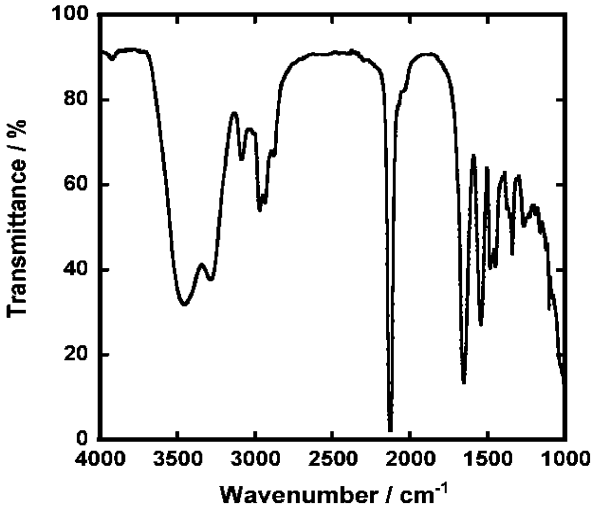


Figure 2. FT IR spectrum of **1**.

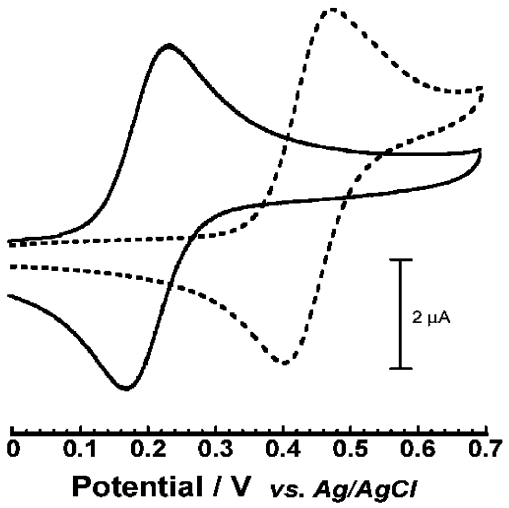


Figure 3. Cyclic voltammogram of 0.1 mM **1** (solid line) or **2** (dotted line) in 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$. The scan rate was 100 mV/s.

Table 1. Synthetic DNAs used in this study

Abbreviation	Sequence
D1	5′-TAA AAA AAA A-3′
D2	5′-GAA AAA AAA A-3′
D3	5′-AAA ATA AAA A-3′
D4	5′-TAA AAA AAA AAA AAA AAA AA-3′
D5	5′-AAA ATA AAA AAA AAA AAA AA-3′
D6	5′-AAA AAA AAA TAA AAA AAA AA-3′
D7	5′-AAA ATA AAA TAA AAA AAA AA-3′
D8	5′-AGG GGT AAG GTT CAT TAG TTG GAA-3′
HS-D8(−)	5′ HS-$(CH_2)_6$-TTC CAA CTA ATG AAC CTT ACC CCT-3′
rUA$_9$	5′-UAA AAA AAA A-3′
D9	5′-AAA AAA AAA A-3′
D10	5′-TTC CAA CTA ATG AAC CTT ACC CCT-3′
D11	5′-TTT TTT TTT TAT TTT TTT TT-3′
D12	5′-TTT TTT TTT TGT TTT TTT TT-3′
D13	5′-TTT TTT TTT TCT TTT TTT TT-3′
D14	5′-TTT TTT TTT TTT TTT TTT TT-3′

of DNA reacts with carbodiimide, the reactivity depends on pH.[47] The pK_a of the imino moiety of thymine or guanine base is 9.9–10.5 or 9.4–10.0, respectively,[52] and therefore, the reactivity of the imino moieties increased with pH and the labeling reaction progressed quantitatively in a short period of time at higher pH. For example, **1** reacted with **D1** quantitatively within 4 h at pH 9.5. On the other hand, **D2** carrying guanine base reacted with **1** faster than that of thymine base (Fig. 6B). However, prolongation of the reaction time resulted in a decrease in the yield. Since the peak of the starting **D1** increased at the expense of the product, the labeled product of **1** at the guanine base should have hydrolyzed under alkaline conditions. Similar behavior was reported in the reaction of carbodiimide with nucleic bases.[47]

In the third step, the reactivity of **1** was tested at 37 or 50 °C. The reaction at 50 °C progressed quantitatively in a shorter period than that at 37 °C as shown in Figure 7. However, extension of the reaction time brought about a decrease in yield, because of hydrolysis of the labeled product at the higher temperature. The same behavior was also observed for **2**. The reactivity of **1** was not influenced by the position of thymine base in the sequence or the length of DNA (compare **D1** with **D3** or **D4** in Fig. 9).

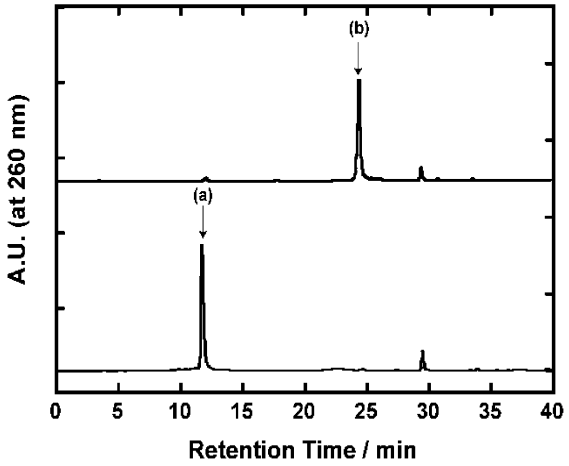


Figure 4. Reversed phase HPLC before (a) and after (b) reaction of 0.5 mM **D1** with 50 mM **1** in 20 mM borate buffer (pH 8.5) containing 30% DMSO at 37 °C for 12 h.

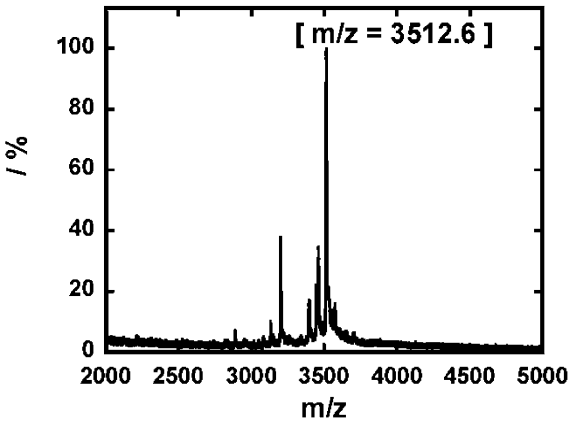


Figure 5. MALDI TOF MS of the HPLC fraction at 23 min in Figure 4. Matrix, 3-HPA; mode, negative. *m/z* [M−H]=3512.6 (theory for $C_{124}H_{152}N_{54}O_{54}P_{10}Fe$, 3512.1).

2.3. Reactivity for RNA

The reactivity of **1** with rUA$_9$ as RNA was also studied. The product was identified by MALDI TOF MS and the

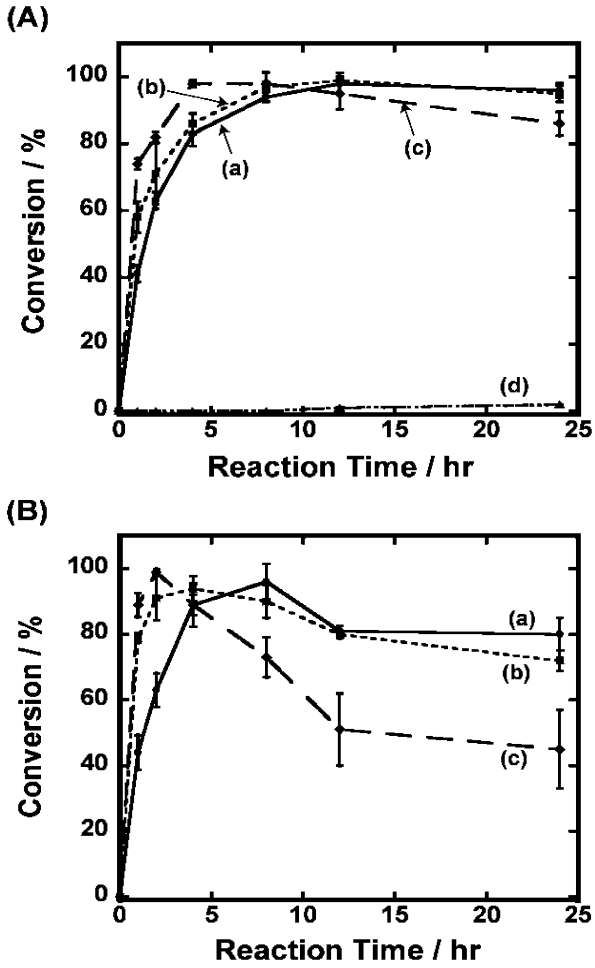


Figure 6. (A) pH dependence of reactivity of 0.5 mM **D1** with 50 mM **1** in 20 mM borate buffer containing 30% DMSO at pH 8.5 (a), pH 9.0 (b) or pH 9.5 (c). The reactivity of 0.5 mM **D9** with 50 mM **1** in 20 mM borate buffer at pH 9.0 is also shown by trace (d). (B) pH dependence of the reactivity of 0.5 mM **D2** with 50 mM **1** in 20 mM borate buffer containing 30% DMSO at pH 8.5 (a) pH 9.0 (b) or pH 9.5 (c). All experiments were conducted at 37 °C.

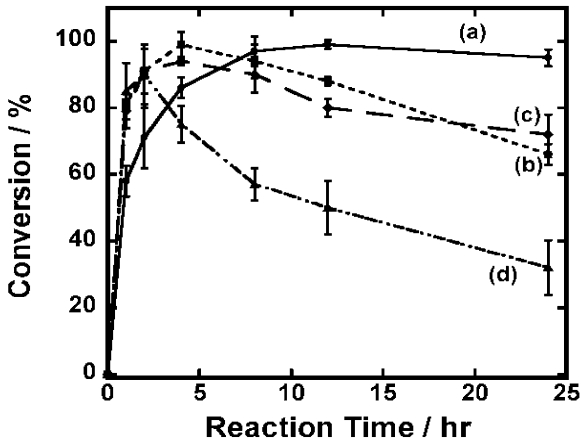


Figure 7. Temperature dependence of the reactivity of 0.5 mM **D1** with 50 mM **1** in 20 mM borate buffer (pH 9.0) containing 30% DMSO at 37 °C (a) or 50 °C (b). Temperature dependence for **D2** is shown for reaction at 37 °C (c) or 50 °C (d).

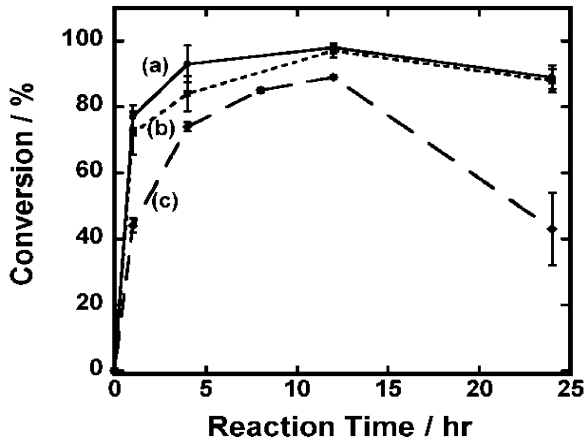


Figure 9. Sequence dependence of the reactivity of 0.5 mM **D3** (a) or **D4** (b) with 50 mM **1** in 20 mM borate buffer (pH 9.5) containing 30% DMSO at 37 °C. The reactivity of 0.1 mM rUA_9 with 10 mM **1** is also shown by trace (c) in the same buffer at pH 8.5.

reactivity was evaluated from the peak intensity of HPLC. A new peak was observed at 23 min with the progress of reaction in addition to the peak of rUA_9 at 9 min. The former was collected and subjected to MALDI TOF MS analysis to reveal a 1:1 adduct of rUA_9 with **1** as shown in Figure 8. Ferrocene-labeling reagent **1** gave the labeled product for rUA_9 in 80% yield in 10 h at pH 8.5 and 37 °C and prolongation of the reaction time resulted in a poorer yield (Fig. 9). Precipitation was observed during the reaction of **1** and rUA_9 at pH 9.5.

2.4. Stability of double stranded DNA labeled with 1

Since the imino moiety of thymine or guanine is involved in the hydrogen bonding of a DNA duplex, its modification with carbodiimide could destabilize the duplex structure of DNA or RNA. Bucci et al. reported the stability of a 17-meric DNA duplex having thymine modified with a ferrocenylmethyl group at the imino moiety.[30] Terminal modification with a ferrocenylmethyl moiety exerted only a small effect on the stability of the duplex, whereas modification in the middle of the sequence brought about larger destabilization by −14 °C as assessed by T_m measurement.

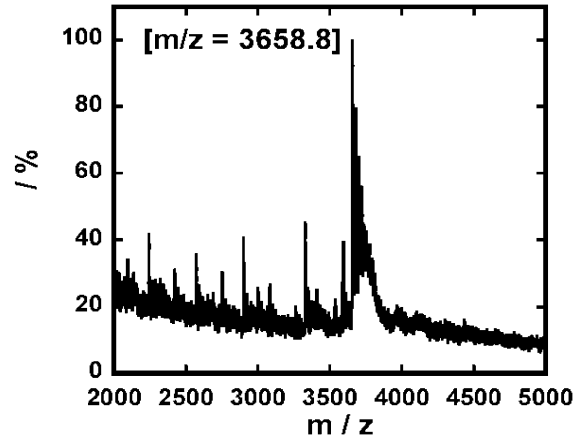


Figure 8. MALDI TOF MS spectrum of rUA_9 after reaction with **1**. Matrix, 3-HPA; mode, negative. *m/z* [M−H]=3658.8 (theory for $C_{123}H_{139}N_{54}O_{64}P_{10}Fe$, 3660.5).

To evaluate the effect of the modification with **1** on the stability of a DNA duplex, **D4**–**D8** modified with **1** and their adducts were purified by HPLC. After hybridization of these modified **D4**–**D8** with their complementary DNAs, T_m was measured in 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0) containing 100 mM KCl (Fig. 10). The T_m values calculated from the melting curves are summarized in Table 2. Terminal modification of **D4** with **1** did not destabilize its DNA duplex appreciably (Fig. 10A, entry 1 in Table 2), whereas middle modification in **D5** and **D6** caused considerable destabilization of their DNA duplexes (entries 2 and 3). Modification of **D7** with **1** at two sites destabilized its DNA duplex further (entry 4). Modification with **1** in the middle destabilized the DNA by −10 °C in T_m values (entries 2 and 3), which is in agreement with the previous paper.[30] Nevertheless, all of the modified DNAs could still form a DNA duplex at low temperature and therefore hybridization was monitored there.

Compound **D8** carrying many thymine and guanine bases in its sequence was also modified with **1** possibly in more than one position. In fact, many peaks including three main peaks were observed in reversed phase HPLC upon reaction of **D8** with **1**. These three peaks were collected separately and analyzed by MALDI TOF MS. It turned out that they were **D8** modified by one, two or three molecules of **1**. The melting curves of the DNA duplex of **D8** labeled with one to three molecules of **1** with its complementary DNA are shown in Figure 10B and entries 5–7 in Table 2. The T_m curve shown in Figure 10B was broader than that in Figure 10A. This is reasonable given the fact that the fraction of **D8** modified by one molecule of **1** could still be a mixture carrying one molecule of **1** in a different position. As the number of modified **1** increased, the T_m values were lowered. Nonetheless, all of these DNA duplexes were stable at 10 °C, as proven by the circular dichroism (CD) spectra of **D8** unlabeled or labeled triply with **1** before and after hybridization with its complementary DNA (Fig. 11).

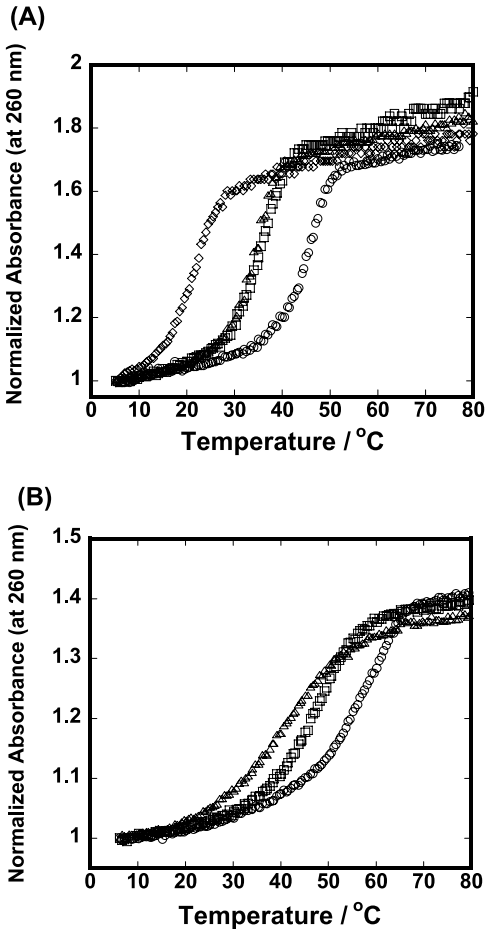


Figure 10. (A) Melting curves of the DNA duplex of 5 μM **D4** (○), **D5** (□), **D6** (△), or **D7** (◇) modified with **1** with respective complementary DNA in 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0) containing 0.1 M KCl. (B) Melting curves of DNA duplex of 5 μM **D8** modified with one (○), two (□), or three (△) molecules of **1** with 5 μM its complementary DNA in 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0) containing 0.1 M KCl.

The effect of introduction of **1** to the thymine base on the stability of the DNA duplex was studied (Table 3). Before the modification, occurrence of a mismatch in the middle of the sequence lowered T_m by ca. 4.0 °C (entries 1–4). Upon modification of thymine with **1**, the T_m was lowered by ca. 10 °C (entry 5), and the magnitude of this T_m lowering was barely dependent on the type of mismatch (entries 6–8), demonstrating that modification in the middle of the sequence with bulky **1** impairs duplex formation to a larger extent than ordinary mismatches. Nonetheless, the fact that the duplex stability is nearly independent of the type of mismatch is advantageous, as hybridization can be carried out under uniform conditions, in this case at low temperature.

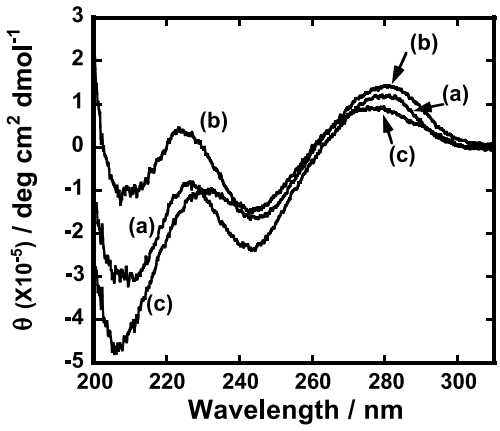


Figure 11. CD spectra of 3 μM **D8** unmodified (a) or modified (b) with three molecules of **1** after hybridization with its complementary DNA in 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0) containing 0.1 M KCl at 10 °C. The CD spectrum of 3 μM **D8** before hybridization is also shown (c).

2.5. Electrochemical detection of D8 labeled with 1 or 2

Electrochemical DNA detection was carried out by using a **HS-D8**(−)-immobilized electrode. Target DNA of **D8** was allowed to react with **1** or **2** and **D8** modified with three molecules of **1** or **2** was used for hybridization reaction (Fig. 12). The SWV method was used in this experiment because of its low background current.[53] The current peak based on the ferrocene moieties of **D8** modified with **1** was observed at 0.20 V and its intensity increased with an increase in the amount of **D8** modified with **1**, whereas no peak current was observed for **D10** modified with three molecules of **1** as negative control (Fig. 12A). This result indicated that **D8** modified with **1** hybridized indeed with the complementary **HS-D8**(−) on the electrode.

Analogously, the current peak was observed at 0.43 V for **D8** modified with **2** and the peak intensity was proportional to its concentration (Fig. 12B). Since the peak currents started to level off above 1 μM **D8** (Figs. 12A and B), the DNA on the electrode seemed to be covered by around 1 μM **D8**. It was estimated from the peak currents that the electrode is covered with 4.9×10^{11}–9.9×10^{12} molecules of 24-meric DNA/cm^2 at saturation. This value is in good agreement with the data previously described by Tarlov and Georgiadis groups.[54–61] In conclusion, the DNA hybridization was quantitative at **D8** concentrations lower than 1.0 μM.

Table 2. Melting temperature of several combinations of DNA duplexes between **1**-modified DNAs and their complementary DNAs

Entry	Sequence[a]	T_m (°C)	ΔT_m (°C)[b]
1	5′-T^{Fc}AA AAA AAA AAA AAA AAA AA-3′ 3′-A TT TTT TTT TTT TTT TTT TT-5′	44.2	−0.9
2	5′-AAA AT^{Fc}A AAA AAA AAA AAA AA-3′ 3′-TTT TA T TTT TTT TTT TTT TT-5′	34.2	−10.5
3	5′-AAA AAA AAA T^{Fc}AA AAA AAA AA-3′ 3′-TTT TTT TTT A TT TTT TTT TT-5′	34.1	−10.3
4	5′-AAA AT^{Fc}A AAA T^{Fc}AA AAA AAA AA-3′ 3′-TTT TA T TTT A TT TTT TTT TT-5′	22.1	−22.2
5	5′-AGG GGT AAG GTT CAT TAG TTG GAA-3′(1Fc) 3′-TCC CCA TTC CAA GTA ATC AAC CTT-5′	55.3	−4.5
6	5′-AGG GGT AAG GTT CAT TAG TTG GAA-3′(2Fc) 3′-TCC CCA TTC CAA GTA ATC AAC CTT-5′	47.8	−12.0
7	5′-AGG GGT AAG GTT CAT TAG TTG GAA-3′(3Fc) 3′-TCC CCA TTC CAA GTA ATC AAC CTT-5′	39.1	−20.7

[a] Fc represents ferrocene of **1** modifying the site(s) marked in the sequence.
[b] $\Delta T_m = T_m$(modified with **1**) − T_m(unmodified).

Table 3. Effect of the type of mismatch on the DNA duplex stability

Entry	Sequence	X	T_m (°C)	ΔT_m (°C)[a]
1	5′-AAA AAA AAA TAA AAA AAA AA-3′ 3′-TTT TTT TTT XTT TTT TTT TT-5′	A	44.4	—
2		G	42.0	−2.4
3		C	40.0	−4.4
4		T	39.8	−4.6
5	5′-AAA AAA AAA T^{Fc}AA AAA AAA AA-3′ 3′-TTT TTT TTT X TT TTT TTT TT-5′	A	34.1	−10.3
6		G	35.2	−9.2
7		C	34.9	−9.5
8		T	36.3	−8.1

[a] $\Delta T_m = T_m$(T/X or T^{Fc}/X) − T_m(T/A).

2.6. Electrochemical gene expression analysis

As a model gene expression experiment was successful with **D8** modified with **1** or **2**, competitive hybridization was attempted with a **HS-D8**(−)-immobilized electrode. **D8** modified with **1** or **2** was mixed at various ratios and allowed to hybridize with the **HS-D8**(−)-immobilized electrode. As shown in Figure 13A, two current peaks were obtained at 0.20 and 0.43 V in SWV measured in 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$. The intensity of these peaks was proportional to the amount of **D8** modified with **1** or **2** and the total peak current was ca. 40 nA, which correspond to ca. 4.0×10^{12} molecules/cm^2. This result shows that **D8** modified with **1** or **2** can hybridize on the electrode competitively.

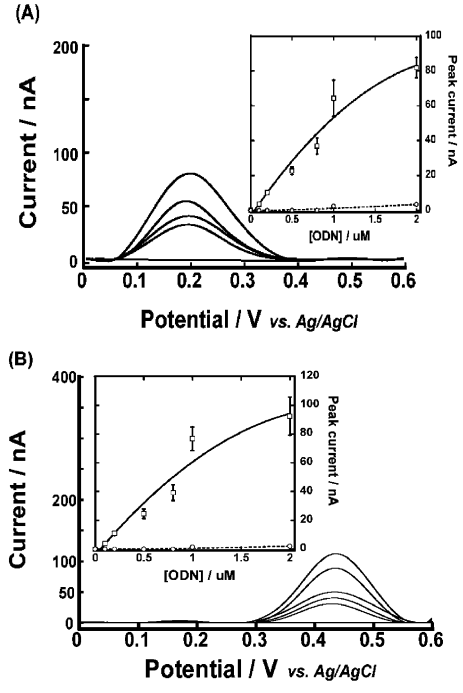


Figure 12. (A) Square wave voltammogram of **HS-D8**(−) immobilized on the electrode after hybridization with different concentrations of **D8** modified with three molecules of **1** (□) or **D10** (○) in 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$ at 10 °C. ΔE_p = 50 mV, ΔE_s = 10 mV, f = 10 Hz. A standard line for the current peak at 0.20 V was plotted against the concentration of **D8**. (B) Square wave voltammogram of **HS-D8**(−) immobilized on the electrode after hybridization with different concentrations of **D8** modified with three molecules of **2** (□) or **D10** (○) in 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$ at 10 °C. ΔE_p = 50 mV, ΔE_s = 10 mV, f = 10 Hz. A standard line for the current peak at 0.43 V was plotted against the concentration of **D8**.

Figure 13B shows a plot of the observed ratio of peak current of **D8** modified with **1** or **2** against the mixed ratio of **D8** modified with **1** or **2**. A good correlation obtained suggested the feasibility of electrochemical expression analysis by using **D8** modified with **1** and **2** coupled with a **HS-D8**(−)-immobilized electrode.

3. Conclusion

Ferrocenylcarbodiimide derivatives **1** and **2** having a different redox potential were designed and synthesized. They could react with DNA or RNA quantitatively under basic conditions. Although the labeling of DNA and RNA with **1** and **2** destabilized their DNA duplex, a DNA duplex could still form at low temperature. The ferrocene-labeled

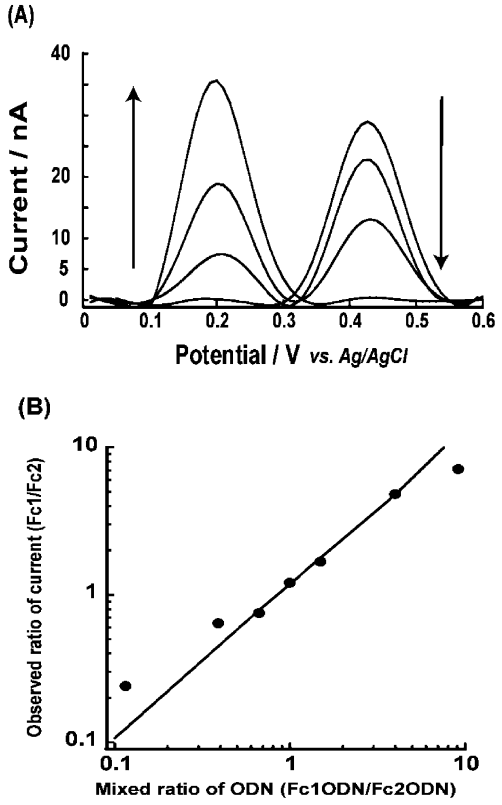


Figure 13. (A) Square wave voltammogram of a **HS-D8**(−)-immobilized electrode after hybridization with a mixture of **D8** modified with **1** or **2** (1.5:0.5, 1.2:0.8, 0.8:1.2, 0.5:1.5 μM/μM) in 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$ at 10 °C. ΔE_p = 50 mV, ΔE_s = 10 mV, f = 10 Hz. (B) Plot of the observed ratio of the peak current of **D8** modified with **1** to that with **2** against the mixed ratio of **D8** modified with **1** to that with **2**.

DNA could hybridize with its complementary DNA probe-immobilized electrode and the peak current was proportional to the amount of the target DNA. When DNA was labeled differentially with **1** or **2**, the resulting DNA gave rise to signals competitively on the electrode, making electrochemical gene expression analysis promising with the detection limit being ca. 0.05 μM DNA sample in 1 μl (ca. 50 fmol).

4. Experimental

4.1. Chemicals

N,*N*-bis(3-aminopropyl)methylamine, *S*-*tert*-butoxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine, ethylisocyanate, ferrocenylcarboxylic acid, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), *p*-toluenesulfonyl chloride (TsCl), and iodomethane were purchased from Tokyo Kasei Co., (Tokyo, Japan). HCl/1,4-dioxane was purchased from Watanabe Chemical Inc. (Hiroshima, Japan). Solvents used in this paper, were purchased from Wako Chemicals Inc. (Osaka, Japan). Ferrocenepropionic acid was synthesized according to the method reported previously.[35] Synthetic DNAs, rUA_9 of RNA, and thiolated DNA were custom-synthesized by Genenet Co., (Fukuoka, Japan). The sequences of these DNAs are listed in Table 1. **D8**(−) carried a sequence complementary to that of mRNA coding for the cytochrome c gene of Xenopus. MilliQ water was used throughout (Millipore, Billerica, MA). Buffers were prepared from the following chemicals: boric acid, disodium hydrogenphosphate dodecahydrate, sodium dihydrogenphosphate dihydrate, dipotassium hydrogenphosphate, potassium dihydrogenphosphate, sodium hydroxide, triethylamine, and hydrochloric acid were purchased from Wako. Tris(hydroxymethyl)aminomethane was purchased from Nacalai Tesque (Kyoto, Japan). Buffers 20×SSC (0.3 M sodium citrate containing 3 M NaCl) and 2×SSC for hybridization were purchased from Wako. Buffer for reaction of **1** and **2** with DNA and RNA was prepared as 50 mM NaH_2BO_3/NaOH buffers (pH 8.5, 9.0 or 9.5) and used after dilution. Two hundred mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0) containing 1 M $NaClO_4$ or 1 M KCl was prepared and used after dilution in electrochemical or spectrophotometric measurements, respectively, and 100 mM triethylammonium acetate (TEAA) buffer (pH 7.0) was used as an eluent in high performance liquid chromatography (HPLC).

4.2. Instruments

4.2.1. Identification of product. Compounds **1** and **2** were characterized mainly by ^{1}H NMR (250 MHz spectrometer, Bruker, Rheinstetten, Germany) and Fourier transform infrared (FT IR, Spectrum One FT IR, Perkin Elmer Co., Wellesley, MA). Tetramethylsilane (TMS) was used as a standard in ^{1}H NMR measurement. IR was measured with 4 cm^{-1} resolution after sandwiching the sample between CaF_2 single crystal plates.

4.2.2. HPLC analysis. The HPLC system used in this experiment, was composed of the following components: Hitachi C-7300 column oven, L-7450H diode array detector, L-7100 pump and D-7000 interface chromatograph. Reversed phase HPLC was run using a Lichrospher RP-18 (Cica-Merck, Kanto Chemicals Co., Tokyo, Japan) column with the gradient condition where the acetonitrile content in 100 mM TEAA buffer (pH 7.0) was linearly changed from 0 to 40% over 30 min at a flow rate of 1.0 ml/min with detection at 260 nm. The reactivity of **1** and **2** with single stranded DNAs or RNA was assessed from the ratio of peak heights for unmodified and modified DNAs or RNA.

4.2.3. MALDI TOF MS analysis. DNAs or RNA modified with **1** or **2** were characterized by matrix-assisted laser desorption ionization time-of-flight mode mass spectrometry (MALDI TOF MS, Voyager™ Linear-SA, PerSeptive Biosystems Inc., Fostercity, CA) measurement of the products separated by HPLC. They were desalted by Dowex 50WX8 cation exchange resin and dissolved in a solution of 50 mg/ml 3HPA (3-hydroxypicolinic acid) in 0.1% TFA/50% CH_3CN and dried. Mass spectra were measured by the negative mode.

4.2.4. Melting curve measurement. Melting curves of DNA duplexes were measured on a Hitachi 3300 spectrometer equipped with an SPR 10 temperature controller. The concentration of DNAs unmodified or modified with **1** or **2** was estimated from the molar absorptivity at 260 nm, 6229 or 6201 $cm^{-1}\,M^{-1}$ for **1** or **2**, respectively. Melting temperature was measured in 20 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0) containing 100 mM KCl. A mixture of 5 μM DNAs modified with **1** or **2** and 5 μM their complementary DNA was placed in the cell of 1 cm in light path length (total 200 μl) and absorbance change at 260 nm was monitored with raising to 85 °C at a rate of 0.5 °C/min.

4.2.5. Electrochemical measurement. Electrochemical measurement was made on an ALS Electrochemical Analyzer Model 900 (CH Instrument Inc., Austin, TX). The redox behavior of 100 μM **1** or **2** was monitored by cyclic voltammetric (CV) measurement over a scan range of 0–0.7 V at a scan rate of 100 mV/s. The Osteryoung square wave voltammetry (SWV) method was used in the experiments for DNA-immobilized electrodes before and after hybridization with an amplitude of 50 mV, applied potential of 10 mV, and frequency of 10 Hz. The electrolyte used was 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$, as it is known that the stable redox reaction of ferrocence occurs in this buffer.[62] The cell was furnished with three electrodes of Ag/AgCl as reference electrode, Pt wire as counter electrode, and DNA-immobilized electrode as working electrode. All measurements were conducted at 10 °C where the double stranded structure of DNA used is stable on the working electrode.

4.3. Synthesis of 1 and 2

Ferrocenylcarbodiimides **1** and **2** were synthesized according to the route shown in Scheme 2.

4.3.1. [3-[(3-Aminopropyl)methylamino]propyl]carbamic acid *tert*-butyl ester (3). *N*,*N*-bis(3-aminopropyl)-methylamine (10 ml, 60 mmol) was dissolved in 20 ml of

1,4-dioxane and a solution of *S-tert*-butoxycarbonyl-4,6-dimethyl-2-mercaptopyrimidine (7.7 g, 30 mmol) in 50 ml of 1,4-dioxane was dropped to the solution over 10 h at room temperature and the mixture was stirred for 20 h. After filtration of the yellow solid, the solvent was removed under reduced pressure and 50 ml of water were added. The white precipitates formed were removed by filtration and NaCl was added to the filtrate. Further precipitates and remaining solid NaCl were removed by filtration and mono Boc derivative **3** was extracted with ethyl acetate (30 ml×4). The extract was dried over magnesium sulfate and **3** was obtained as a yellow oil (4.7 g, 32% yield) after evaporation and drying under reduced pressure. ^{1}H NMR (250 MHz, $CDCl_3$) δ=1.44 (9H, s, $C(CH_3)_3$), 1.66 (4H, m, CH_2CH_2-CH_2), 2.19 (3H, s, NCH_3), 2.36 (4H, m, NCH_2CH_2), 2.75 (2H, m, $CH_2CH_2NH_2$), and 3.16 (2H, m, CH_2CH_2NHCO) ppm.

4.3.2. [3-[[3-(3-Ethylureido)propyl]methylamino]propyl]carbamic acid *tert*-butyl ester (4). A solution of ethylisocyanate (1.5 g, 20 mmol) in 13 ml of diethyl ether was added slowly to a solution of **3** (4.7 g, 9.0 mmol) in 7 ml of diethyl ether at 0 °C with stirring. The reaction mixture was stirred for 3 h at room temperature after standing for 10 min at 0 °C. The mixture became negative to ninhydrin in 3 h and the solvent was removed and the residue dried under reduced pressure to give **4** as a yellow oil (5.5 g, 95% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ=1.11 (3H, t, $NHCONHCH_2CH_3$), 1.45 (9H, s, $C(CH_3)_3$), 1.64 (4H, m, $CH_2CH_2CH_2$), 2.18 (3H, s, NCH_3), 2.37 (4H, m, NCH_2CH_2), and 3.18 (6H, m, CH_2CH_2NHCO, CH_2CH_2-$NHCOO$(tBu), $CONHCH_2CH_3$) ppm.

4.3.3. 1-[3-[(3-Aminopropyl)methylamino]propyl]-3-ethylurea (5). Compound **4** (5.5 g, 17 mmol) was dissolved in 4 N HCl/1,4-dioxane (13 ml, 52 mmol) and stirred for 3 h. The precipitates formed were collected and dried under reduced pressure to give **5** as a yellow gum-like solid (5.2 g, 95% yield). ^{1}H NMR (250 MHz, DMSO-d_6) δ=1.00 (3H, t, $NHCONHCH_2CH_3$), 1.80 (2H, m, CH_2CH_2NHCO), 2.03 (2H, m, $CH_2CH_2N^+H_3$) 2.70 (3H, s, NCH_3), and 3.03 (10H, m, $N^+HCH_2CH_2$, $CH_2CH_2N^+H_3$, CH_2CH_2NHCO, $CONHCH_2CH_3$) ppm.

4.3.4. *N*-[3-[[3-(3-Ethylureido)-propyl]-methylamino]-propyl]-3-ferrocenylpropionamide (6). Compound **5** (2.5 g, 8.8 mmol) was dissolved in TEA (5.1 ml, 36 mmol) and chloroform (30 ml). Ferrocenepropionic acid (2.6 g, 10 mmol), PyBOP (5.2 g, 10 mmol), and HOBt (1.3 g, 10 mmol) were added to the solution and stirred for 10 h at room temperature. The progress of reaction was monitored by the spot of R_f=0.22 on thin-layer chromatography (TLC) ($CHCl_3/CH_3OH$/TEA=95:5:1) on silica gel. The solution was washed with saturated $NaHCO_3$ aqueous solution (30 ml×2) and the solvent was removed under reduced pressure. After collection of the R_f=0.22 fraction on silica gel chromatography with the eluent ($CHCl_3/CH_3OH$/TEA=95:5:1), compound **6** was obtained as a pale yellow oil (2.8 g, 70% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ=1.11 (3H, t, $NHCONHCH_2CH_3$), 1.80 (4H, q, $CH_2CH_2CH_2$), 2.14 (3H, s, NCH_3) 2.38 (4H, m, CH_2NCH_3), 2.68 (2H, t, $FcCH_2CH_2$), 3.02–3.35 (8H, m, $FcCH_2CH_2$, CH_2NHCO), and 4.12 (9H, m, $C_5H_5FeC_5H_4CH_2$) ppm.

4.3.5. *N*-[3-[[3-(3-Ethylureido)-propyl]-methylamino]-propyl]-3-ferrocenylamide (7). Compound **5** (2.5 g, 8.8 mmol) was dissolved in TEA (5.1 ml, 36 mmol) and chloroform (30 ml), ferrocenecarboxylic acid (2.3 g, 10 mmol), PyBOP (5.2 g, 10 mmol), and HOBt (1.3 g, 10 mmol) were added to the solution and stirred for 18 h at room temperature. The progress of reaction was monitored by the spot of R_f=0.20 on TLC ($CHCl_3/CH_3OH$/TEA=95:5:1). The reaction mixture was washed with saturated $NaHCO_3$ aqueous solution (30 ml×2) and the solvent was removed under reduced pressure. The yellow fraction of R_f=0.20 ($CHCl_3/CH_3OH$/TEA=95:5:1) was collected from silica gel chromatography. After removing the solvent and drying under reduced pressure **7** was obtained as a pale yellow oil (1.9 g, 51% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ=1.11 (3H, t, $NHCONHCH_2CH_3$), 1.80 (4H, q, CH_2CH_2-CH_2), 2.14 (3H, s, NCH_3) 2.38 (4H, m, CH_2NCH_3), 3.05–3.43 (6H, m, CH_2NHCO), 4.15 (5H, s, $(C_5H_5)Fe(C_5H_4)$-CONH), 4.33 (2H, m, $(C_5H_5)Fe(C_2H_2C_2H_2C)CONH$), and 4.63 (2H, m, $(C_5H_5)Fe(C_2H_2C_2H_2C)CONH$) ppm.

4.3.6. *N*-[3-[(3-Ethyliminomethyleneaminopropyl) methylamino]propyl]-3-ferrocenylpropionamide (8). The reaction was carried out under the nitrogen atmosphere. Compound **6** (2.0 g, 4.4 mmol) was dissolved in TEA (2.4 ml, 18 mmol) and dry dichloromethane (20 ml) and stirred for 15 min. The solution was kept for 15 min at −20 °C. A dry dichloromethane solution (15 ml) of *p*-toluenesulfonyl chloride (1.7 g, 8.8 mmol) was added to the solution slowly. After standing at room temperature, the reaction mixture was refluxed for 4 h and the progress of reaction was monitored by the spot of R_f=0.48 on TLC ($CHCl_3$/TEA=100:0.5). The reaction mixture was washed with 40% potassium carbonate aqueous solution (20 ml×4) and the solvent was removed. The solid obtained was dissolved in 30 ml of diethyl ether and insoluble material was removed by filtration. The solvent was evaporated under reduced pressure and the residue was dried to give **8** as an orange viscous oil (0.81 g, 41% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ=1.23 (3H, t, $N{=}C{=}NCH_2CH_3$), 1.68 (4H, m, $CH_2CH_2CH_2$), 2.16 (3H, s, NCH_3) 2.38 (4H, m, CH_2NCH_3), 2.68 (2H, t, $FcCH_2CH_2$), 3.32–3.49 (8H, m, $FcCH_2CH_2$, $CH_2N{=}C{=}N$, CH_2NHCO), and 4.10 (9H, m, $C_5H_5FeC_5H_4CH_2$) ppm.

4.3.7. *N*-[3-[(3-Ethyliminomethyleneaminopropyl) methylamino]propyl]-3-ferrocenylamide (9). The reaction was carried out under the nitrogen atmosphere. Compound **7** (1.5 g, 3.5 mmol) was dissolved in TEA (1.9 ml, 14 mmol) and dry dichloromethane (15 ml) and stirred for 15 min. The solution was kept for 15 min at −20 °C. A dry dichloromethane solution (10 ml) of *p*-toluenesulfonyl chloride (1.3 g, 7.0 mmol) was added to the solution slowly. After standing at room temperature, the reaction mixture was refluxed for 4 h. The progress of reaction was monitored by the spot of R_f=0.42 on TLC ($CHCl_3$/TEA=100:0.5). The reaction mixture was washed with 40% potassium carbonate aqueous solution (20 ml×4) and the solvent was removed. The solid left was dissolved in 30 ml of diethyl ether and insoluble material was removed

by filtration. The solvent was evaporated under reduced pressure and the residue was dried to give **9** as an orange viscous oil (0.55 g, 38% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ=1.11 (3H, t, N=C=NCH$_2$C*H*$_3$), 1.70 (4H, q, CH$_2$C*H*$_2$CH$_2$), 2.14 (3H, s, NC*H*$_3$) 2.38 (4H, m, C*H*$_2$NCH$_3$), 3.02–3.35 (6H, m, C*H*$_2$N=C=N, C*H*$_2$NHCO), 4.15 (5H, s, (C$_5$*H*$_5$)Fe(C$_5$H$_4$)CONH), 4.33 (2H, m, (C$_5$H$_5$)Fe(C$_2$*H*$_2$C$_2$H$_2$C)CONH), and 4.63 (2H, m, (C$_5$H$_5$)Fe(C$_2$H$_2$C$_2$*H*$_2$C)CONH) ppm.

4.3.8. (3-Ethyliminomethyleneaminopropyl)dimethyl[3-(3-ferrocenylpropionylamino)propyl]ammonium iodide (1). Compound **8** (0.81 g, 1.8 mmol) was dissolved in diethyl ether (3 ml) and iodomethane (1.1 ml, 3.6 mmol) was added and stirred for 18 h. The precipitates formed were collected by filtration and dried under reduced pressure to give **1** as a yellow solid (0.76 g, 72% yield). ^{1}H NMR ($CDCl_3$) δ=1.23 (3H, t, N=C=NCH$_2$C*H*$_3$), 1.96–2.16 (4H, m, CH$_2$C*H*$_2$CH$_2$), 2.68 (2H, m, FcC*H*$_2$CH$_2$), 3.21 (6H, s, N$^+$(C*H*$_3$)$_2$), 3.24–3.48 (12H, m, C*H*$_2$N$^+$(CH$_3$)$_2$, FcCH$_2$-C*H*$_2$, C*H*$_2$N=C=N, C*H*$_2$NHCO), and 4.10 (9H, m, C$_5$*H*$_5$FeC$_5$*H*$_4$CH$_2$) ppm, FT IR (CaF_2) 2129 cm^{-1} (–N=C=N–), 1643 cm^{-1} (–NH–CO–), 1559 cm^{-1} (–NH–CO–).

4.3.9. (3-Ethyliminomethyleneaminopropyl)dimethyl[3-(3-ferrocenylamino)propyl]ammonium iodide (2). Compound **9** (0.55 g, 1.3 mmol) was dissolved in diethyl ether (3 ml) and iodomethane (0.80 ml, 2.6 mmol) was added and stirred for 18 h. The precipitates formed were collected by filtration and dried under reduced pressure to give **2** as a yellow solid (0.49 g, 68% yield). ^{1}H NMR ($CDCl_3$) δ=1.24 (3H, t, N=C=NCH$_2$C*H*$_3$), 1.93–2.14 (4H, m, 2×CH$_2$-C*H*$_2$CH$_2$), 3.19 (6H, s, N$^+$(C*H*$_3$)$_2$), 3.20–3.49 (10H, m, C*H*$_2$N$^+$(CH$_3$)$_2$, C*H*$_2$N=C=N, C*H*$_2$NHCO), 4.19 (5H, s, (C$_5$*H*$_5$)Fe(C$_5$H$_4$)CONH), 4.42 (2H, m, (C$_5$H$_5$)Fe(C$_2$*H*$_2$C$_2$H$_2$C)CONH), and 4.75 (2H, m, (C$_5$H$_5$)Fe(C$_2$H$_2$C$_2$*H*$_2$C)CONH) ppm, FT IR (CaF_2) 2127 cm^{-1} (–N=C=N–), 1642 cm^{-1} (–NH–CO–), 1557 cm^{-1} (–NH–CO–).

4.4. Labeling reaction of DNAs with 1 or 2

Ten microliters of a solution of 1 mM DNA in 20 mM borate buffer (pH 8.5, 9.0 or 9.5) were mixed with 10 μl of a solution of 100 mM **1** or **2** in 20 mM borate buffer containing 60% DMSO at proper temperature for a specified period of time. The mixture was diluted to 1 ml with 0.1 M TEAA buffer (pH 7.0) and then loaded on a NAP-10 column (Pharmacia Sephadex G-25, Amersham Biosciences Co., Uppasala, Sweden). After discarding the first 1 ml of the flow-through, DNA was eluted with 1.5 ml of 0.1 M TEAA buffer and lyophilized. After addition of 100 μl of water, 20 μl of this solution were subjected to reversed phase HPLC to evaluate the composition.

4.5. Labeling reaction of RNA with 1 or 2

Ten microliters of a solution of 0.2 mM rUA$_9$ in 20 mM borate buffer (pH 8.5) were mixed with 10 μl of a solution of 20 mM **1** or **2** in 20 mM borate buffer containing 60% DMSO at 37 °C for a specified period of time. The mixture was diluted to 1 ml with 0.1 M TEAA buffer (pH 7.0) and then loaded on a NAP-10 column. After discarding the first 1 ml of the flow-through, RNA was eluted with 1.5 ml of 0.1 M TEAA buffer and lyophilized. After addition of 100 μl of water, 20 μl of this solution were subjected to reversed phase HPLC to assess the composition.

4.6. Preparation of a DNA-immobilized electrode and its hybridization with complementary DNA

A gold electrode having 2.0 mm^2 in area was polished with 6 μm, 1 μm of diamond slurry, and 0.05 μm of alumina slurry in this order and sonicated in MilliQ water for 10 min. This electrode was electrochemically polished by scanning 40 times from −0.2 to 1.5 V at a scan rate of 100 mV/s in 1 M H_2SO_4 aqueous solution and sonicated in MilliQ water for 15 min. One microliter of 1 M NaCl solution containing 2 μM thiolated DNA (see Table 1) was placed on the gold electrode held upside down and kept in a closed container under high humidity for 24 h at room temperature. After washing with MilliQ water, 1 μl of 1 mM 6-mercaptohexanol was placed on the electrode for 1 h at 45 °C.

One microliter of 2×SSC containing 0.1, 0.2, 0.5, 0.8, 1 or 2 μM DNA modified with **1** or **2** was placed on the electrode for 6 h at 10 °C to allow hybridization to proceed. The electrode was kept in 20 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) containing 100 mM $NaClO_4$ and SWV was measured with an ALS model 900 Electrochemical Analyzer. Competitive hybridization of two different DNA samples was carried out as follows. One microliter of a mixture of DNAs modified with **1** or **2** (1.8:0.2, 1.5:0.5, 1.2:0.8, 1.0:1.0, 0.8:1.2, 0.5:1.5, or 0.2:1.8 μM/μM) was placed on the DNA-probe immobilized electrode for 8 h at 10 °C to allow hybridization to proceed. These electrodes were dipped in the same electrolyte as above for 1 min and SWV was measured at 10 °C.

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