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Synthesis and DNA binding behavior of a naphthalene diimide derivative carrying two dicobalt hexacarbonyl complexes as an infrared DNA probe

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ABSTRACT

Naphthalene diimide having two dicobalt hexacarbonyl complexes at its substituent termini **1** was designed and synthesized as an infrared probe for double stranded DNA. Spectrophotometric and viscometric DNA binding studies of **1** and naphthalene diimide having two acetylene moieties **2** as its precursor were carried out to prove their threading intercalation binding modes. Fourier Transform Infrared Reflection–Absorption Spectroscopy (FT-IR RAS) measurements were achieved for DNA-immobilized on a gold surface before and after hybridization with complementary DNA strand. Distinguished absorption peaks assigned to the dicobalt hexacarbonyl complexes were obtained in the case of the formation of double stranded DNA after treatment with **1**. This result revealed that **1** can be used as an infrared probe for monitoring double stranded DNA.

1. Introduction

Infrared (IR) analysis is not only applied for identification of organic compounds, but also for identification of secondary structure of protein and DNA [1,2], quantification of sugar or protein in blood plasma or serum [3,4], and imaging of cell or microorganism [5,6]. Although IR is useful for non-labeling analysis of living organisms, IR probes are also important in order to extend this technique for biological applications [7–10]. As one of the example, IR probe was used for internal control to normalize IR signals between different samples because of its variability in repeatability and accuracy tests driven from the difference in the thickness of sample. Potassium thiocyanate (KSCN) [7] or azide (N_3) modified peptide [8] are useful as the internal control for such purpose because of their characteristic stretching vibrations around 1880–2150 cm^{-1}, which are typical for triple bond, and a spectral window for living organism in this range. Quantitative analysis of the components in blood and serum was achieved by the addition of the identified concentration of KSCN in these samples [7]. As another example, Jaouen and co-worker reported a carbonylmetalloimmunoassay (CMIA) by using metal–carbonyl complex [9–13]. Since the metal–carbonyl complex has absorption around 1800–2150 cm^{-1} with strong intensity, high sensitive immunoassay was achieved quantitatively with low background. These studies revealed that development of IR labeling reagent will help in biological application of IR analysis.

In the meantime, we have been developing naphthalene diimide derivatives having ferrocene moieties as electrochemical labels for the detection of double stranded DNA. Ferrocenylnaphthalene diimides were used as electrochemical hybridization indicators [14–19]. These compounds led us to develop naphthalene diimide derivative having metal–carbonyl complex, which can be applied for the IR imaging of DNA in biological system. In this paper, we report on the synthesis of naphthalene diimide having two dicobalt hexacarbonyl complexes at its substituent termini **1** (Scheme 1). The compound **1** and precursor naphthalene diimide having two acetylene moieties **2** were studied in order to evaluate DNA binding affinity using spectroscopic and viscometric measurements. Furthermore, **1** was applied for detection of double stranded DNA on the gold surface by using FT-IR RAS.

2. Experimental

2.1. Reagents and apparatus

^{1}H NMR spectra were recorded on a Bruker AC250P spectrometer operating at 250 MHz or Bruker AVANCE 400 spectrometer operating at 400 MHz for proton with tetramethylsilane (TMS) as an internal standard. Mass (MS) spectra were taken on a Voyager™ Linear-SA (PerSeptive Biosystems Inc., Foster City, CA) by the time-of-flight mode with α-cyano-4-hydroxycinnamic acid as matrix. Fast atom bombardment mass (FAB-MS) spectra were measured by JMS-SX102A (JEOL Ltd., Japan) with dithiodiethanol as matrix. Melting points were uncorrected. Electronic absorption spectra were recorded with a Hitachi 3300 spectrophotometer equipped

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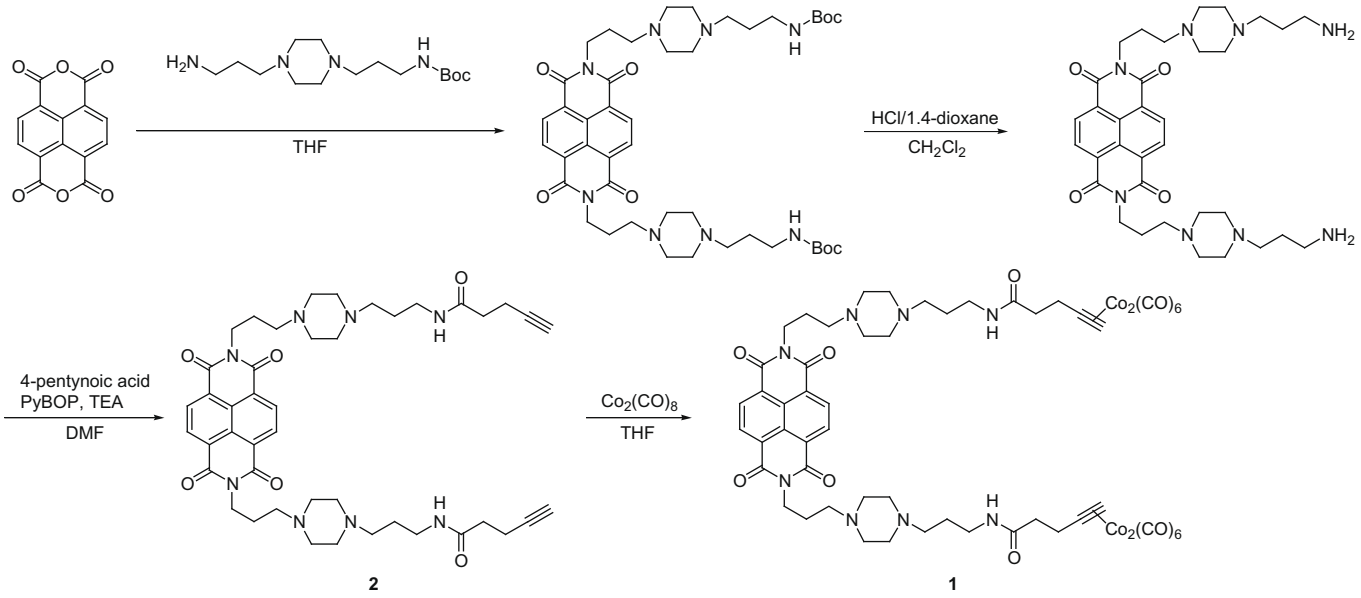

Scheme 1. Synthetic route of compound **1**.

with an SPR10 temperature controller. The high performance liquid chromatography (HPLC) system used in this experiment was composed of the following components: a Shimadzu CTO-10A column oven, a SPD-M10A diode array detector, a LC10AD pump and a SCL-10A interface chromatograph. Reversed-phase HPLC was run using an Inertsil ODS-3 (GL Sciences Co., Tokyo, Japan) column with gradient conditions, where the methanol content in 0.1% trifluoroacetic acid was linearly changed from 40% to 100% over 40 min at a flow rate of 1.0 ml/min with detection at 383 nm.

Dicobalt octacarbonyl was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Morpholinoethanesulfonic acid (MES) and 20 × SSC (0.3 M sodium citrate and 3.0 M sodium chloride) were purchased from Dojindo Lab. (Kumamoto, Japan) and Wako Chemicals Inc. (Osaka, Japan), respectively. Gold substrate (25 × 75 mm) obtained from Nalge Nunc International (Rochester, NY) was used. 2-Mercaptoethanol [ME], 6-merocaptohexanol [MH], and 11-mercaptoundecanol [MU] were purchased from Sigma–Aldrich Japan (Tokyo, Japan). (1-Mercaptoudec-11-yl)-tri(ethylene glycol) [MU(eg)$_3$] was purchased from Toyobo Co. Ltd. (Tokyo, Japan).

Calf thymus DNA was purchased from Sigma–Aldrich (St. Louis, MO) and used after sonication according to the method reported previously [20]. The concentration of calf thymus DNA was estimated from the molar absorptivity based on nucleic bases of 6412 cm^{-1} M^{-1} at 260 nm [21]. Oligonucleotide (5′-TCA GGT TAA CGG ATC AGC TT-3′) and thiolated one (5′-HS-$(CH_2)_6$-TTT TTT TTT TAA GCT GAT CCG TTA ACC TGA-3′) used in this study were custom synthesized by Genenet Co. (Fukuoka, Japan) and their concentrations were estimated from the molar absorptivities based on strand of 2,22,000 and 3,18,000 $cm^{-1}M^{-1}$, respectively.

2.2. Synthesis

Adduct **1** of naphthalene diimide with dicobalt hexacarbonyl complexes was synthesized according to the route shown in Scheme 1.

2.2.1. *N,N′-bis[(4-pentynamidopropyl)piperazin-1-yl]propyl] naphthalene-1,4,5,8-tetra-carboxylic acid diimide (**2**)*

N,N′-bis[[3-(3-aminopropyl)piperazin-1-yl]propyl]naphthalene-1,4,5,8-tetracarboxylic acid diimide was synthesized according to the procedure described previously [22]. The naphthalene diimide have two amino termini (5.22 g, 6.13 mmol) and 4-pentynoic acid (1.80 g, 18.4 mmol) was dissolved in DMF (50 ml). After addition of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 9.57 g, 18.4 mmol) and triethylamine (5.17 ml, 36.8 mmol), the mixture was stirred at room temperature overnight. The solvent was removed and the residue was taken up in chloroform and washed with saturated sodium bicarbonate. The organic layer was dried over magnesium sulfate. The solvent was removed and the residue was chromatographed on silica gel (silica gel 60N, Kanto Chemical Co.) with chloroform/methanol/diethylamine = 10/0.2/0.5 as eluent. The fraction showing an R_f of 0.47 on TLC with the same solvent was collected and the solvent was removed under reduced pressure, leaving 3.94 g of yellow solid (81% yield). Mp 180–181 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.65 (m, 4H), 1.97 (m, 10H), 2.35 (m, 12H), 2.53 (m, 16H), 3.33 (t, J = 6 Hz, 4H), 4.28 (t, J = 7.3 Hz, 4H), 7.17 (br s, 2H) and 8.73 (s, 4H) ppm. Elemental Analysis: Anal. Calc. for $C_{44}H_{56}N_8O_6$: C, 66.64; H, 7.12; N, 14.13. Found: C, 66.26, H, 7.08; N, 14.20%. TOF-MS *m/z* $[M+H]^+$ 794.46 (theory for $C_{44}H_{56}N_8O_6 + H^+$ 792.43).

2.2.2. *N,N′-bis[[hexacarbonyldicobalt(4-pentynamidopropyl) piperazin-1-yl]propyl]naphthalene-1,4,5,8-tetracarboxylic acid diimide (**1**)*

Complexation of **2** with dicobalt hexacarbonyl was carried out according to the literature [23]. A solution of **2** (240 mg, 0.30 mmol) in tetrahydrofuran (5 ml) was stirred in a brown-colored pear-shaped flask after addition of $Co_2(CO)_8$ (380 mg, 1.1 mmol) under argon atmosphere at room temperature. The progress of the reaction was monitored by reversed-phase HPLC. After the completion of the reaction, the solid formed was removed by filtration. Water (400 ml) was added to the filtrate and the solid formed was filtered and dried under reduced pressure to give 0.30 g of **1** in 73% yield. The obtained compound was identified with ^{1}H NMR and FT-IR (KBr pellet). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (m, 4H), 1.57 (m, 4H), 1.70 (m, 8H), 2.65 (m, 24H), 3.28 (m, 4H), 4.20 (m, J = 7.5 Hz 4H), 5.96 (s, 2H), 7.37 (br s, 2H), and 8.69 (s, 4H) ppm.

MS (FAB$^+$, Xe, dithiodiethanol) *m/z* (%) $[M+H]^+$, 1365 (5), 1029 (95), 912 (100), 794 (49).

2.3. Viscomentric measurements

Viscometric titration was carried out with a PC-controlled automatic system (Lauda, Lauda-Konigshofen, Germany) equipped with a capillary Ubbelohde-type viscometer, an automatic pump/stop-watch unit and a thermostatted water bath at 30 ± 0.1 °C. Two micro liter of 1 mM **1** or **2** were added to 50 μM [poly (dA-dT)]$_2$ in 10 mM MES buffer (pH 6.25) and 100 mM NaCl (1 mM EDTA was also present in the case of **2**) by means of a micro syringe without removing the solution from the viscometer. The relative viscosity ratios of DNA alone and its complex with **1** or **2** were calculated using the equation $\eta/\eta_o = (t - t_o)/(t_{DNA} - t_o)$, where t_o is the flow time of the buffer; t and t_{DNA} are the flow times of DNA sample in the presence and absence of **1** or **2**, respectively.

2.4. Determination of binding and dissociation rate constant

The binding constant of ligand **1** or **2** for sonicated calf thymus DNA was determined by Scatchard analysis using condition probability method of McGhee and von Hippel [24] shown below: $r/L = K(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1}$, where r is the moles of ligand bound per base pair, L is the free ligand concentration, K is observed binding constant, and site size n is the number of base pairs occupied by a ligand molecule.

Stopped-flow measurement was carried out in an SF-61 DX2 double mixing stopped flow system (Hi-Tech Scientific Inc., Salisbury, UK) equipped with a Lauda RF206 temperature controller. The dissociation rate constant (k_d) of **1** or **2** from calf thymus DNA was determined by sodium dodecylsulfate (SDS)-driven dissociation method described previously [25]. Eighty microliter of the mixture of 100 μM DNA and 10 μM **1** or **2** and 1% SDS were mixed instantaneously using a piston, and the change in the absorption at 383 nm was measured immediately after mixing. In this condition, most of **1** or **2** was formed as a complex with DNA. When the DNA-**1** or **2** complex is mixed with an SDS solution, the **1** or **2** released is incorporated into the SDS micelles. Since this process is diffusion-controlled, the entire absorption change represents the k_d-dependent process and, therefore fitting of the kinetic trace provided k_d values.

2.5. FT-IR RAS measurements

FT-IR RAS experiments were carried out on a Perkin Elmer Spectrum One FT-IR spectrometer equipped with a Refractor 2™ (Harrick Scientific Inc.) with a single Brewster's angle silicon polarizer plate (grazing angle, 75°), and a liquid-nitrogen cooled mercury cadmium telluride detector (Perkin Elmer). Spectra were taken at room temperature under dried air with aqusitions (128 scan integrating number of 128 times) at a resolution of 4 cm^{-1}.

2.6. Optimization of suitable masking reagent

A gold substrate was treated with a Piranha solution (7:3 = conc. H_2SO_4:30% H_2O_2) twice for 15 min each and washed with MilliQ water. *Caution*: Because the Piranha solution reacts violently, even explosively, with significant quantities of organic material. This substrate was dipped in methanol containing 1 mM ME, MH, MU, or MU(eg)$_3$ and left for 1 h at room temperature. After washing with methanol and MilliQ water, the surface was dried by spin drier and subsequently blowing N_2 gas. An aqueous solution of 100 μM **1** (150 μl) was placed on these masked substrates, kept for 15 min and washed with MilliQ water. After drying with a spin drier, FT-IR RAS of these substrate were measured.

2.7. Preparation of DNA-immobilized gold substrates

A gold substrate was treated with a Piranha solution for 15 min twice and washed with MilliQ water. A solution of 10 μM thiolated oligonucleotide in 2 × SSC (150 μl) was put on the substrate and kept for 18 h at 25 °C. After washing with MilliQ water and drying by blowing N_2 gas, a solution of 1 mM MU(eg)$_3$ was placed on the substrate, kept for 1 h at 25 °C, and subsequently washed with ethanol and dried by a spin drier.

2.8. FT-IR RAS measurements of oligonucleotide-immobilized gold substrates before and after hybridization with complementary oligonucleotide

A solution of 100 μM **1** in 10 mM MES buffer (pH 6.25) and 100 mM NaCl (150 μl) was placed on a DNA-immobilized substrate and kept for 5 min at room temperature. After washing with 200 μl MilliQ water and dried by a spin drier, FT-IR RAS was measured. For hybridization assay a solution of 10 μM complementary oligonucleotide in 2 × SSC (150 μl) was placed on the DNA-immobilized substrate and incubated at 25 °C for 1 h and washed with MilliQ water and dried by spin drier. A solution of **1** was placed on the substrate at room temperature for 5 min and washed with 200 μl MilliQ water and dried by spin drier and FT-IR RAS measurements were carried out.

3. Results and discussion

3.1. Synthesis of naphthalene diimide having dicobalt hexacarbonyl complexes (*1*)

Naphthalene diimide having dicobalt hexacarbonyl complexes (**1**) was synthesized according to Scheme 1. The progress of the coupling of **2** with dicobalt hexacarbonyl complexes was monitored by reversed-phase HPLC (Fig. 1). After 30 min, two peaks were observed at 24 and 34 min at the expense of the peak of **2** at 13 min and the peak at 24 min coalesced into the one at 34 min after an additional 30 min. This result suggested that the peaks at 24 and 34 min were based on naphthalene diimides carrying mono and bis dicobalt hexacarbonyl complexes, respectively, as confirmed by MS spectrum of **1**. A result of FT-IR measurement

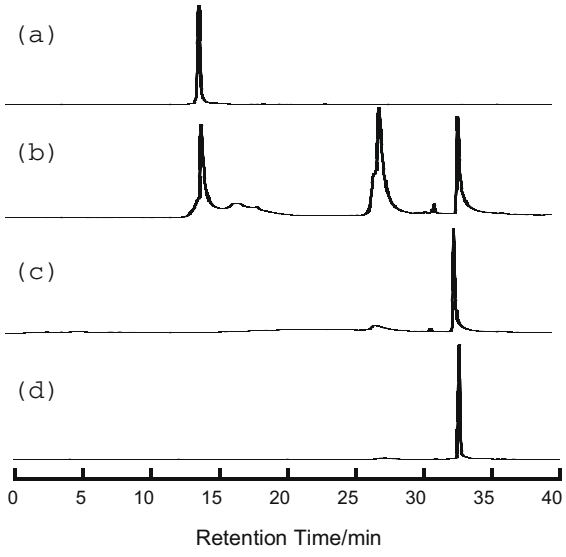

Fig. 1. Reversed-phase HPLC of **2** before (a) and after 30 min (b), and 60 min (c) reaction with $Co_2(CO)_8$. (c). Chromatogram of purified **1** (d).

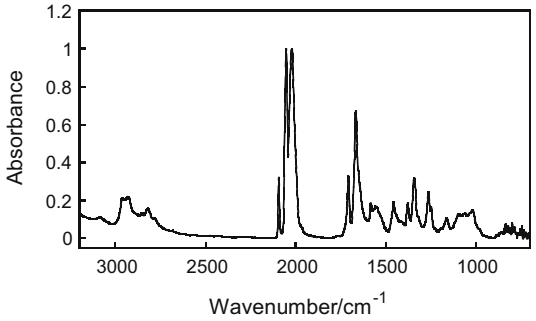

Fig. 2. FI-IR spectra of **1**.

was shown that compound **1** gives rise to absorption maxima at 2092, 2051, and 2021 cm^{-1} characteristic of the carbonyl stretching vibration [23]. Additionally, methylene symmetric and asymmetric stretching vibration at 2800–3000 cm^{-1} and naphthalene diimide C═O, amide C═O stretching vibration and amide N–H bending vibration at 1500–1700 cm^{-1} are observed (Fig. 2). Furthermore, in ^1H NMR spectra the peak at 1.82 ppm based on alkyne protons of **2** shifted downfield at 5.96 ppm upon complexation with the dicobalt hexacarbonyl (**1**) in agreement with the result reported previously [23].

3.2. Affinity of *1* and *2* to double stranded DNA

To evaluate the performance of **1** as an IR hybridization probe, the binding affinities of **1** and **2** for double stranded DNA were studied. Fig. 3 showed the absorption spectra of **1** and **2** in 10 mM MES buffer (pH 6.25) and 100 mM NaCl at 25 °C. The absorption maxima observed for both compounds at 383 nm showed a large hypochromic effect with a small red shift upon addition of calf thymus DNA and these changes were in agreement with the behavior of threading intercalators reported previously [26]. Since an isosbestic point was observed during these spectral changes, the binding constants could be evaluated by Scatchard analysis using McGhee and von Hippel equation [24,27,28] (Fig. 4 and Table 1). Thus, binding constants of 4.5×10^4 and $3.7 \times 10^5\ M^{-1}$ for **1** and **2**, respectively were obtained with the same *n* value of 2, which is similar to that for the naphthalene diimide derivatives carrying bulky substituents such as ferrocene [14,29], tris bipyridine Ru complex [30], or $Os(bpy)_2Cl^+$ [31]. These probes are supposed to undergo threading intercalation into double stranded DNA.

Intercalation of **1** into double stranded DNA was confirmed by viscometric titration of $[poly(dA\text{-}dT)]_2$ with **1**. As shown in Fig. 5, the viscometric titration of a DNA solution increased linearly with an increase in the amount of **1** and the slope for **1** is smaller than that for **2**. This difference is not clear now but, they should come from the difference of the bulkiness of substituent of **1** and **2**, because the viscosity increase is not known to cause by unwinding of DNA duplex intercalated by the intercalator, but also affect by the substituent size of the intercalator or the DNA structure after the intercalator binding [32]. In conclusion, this phenomenon is explained in terms of intercalation of **1** into DNA duplex.

Table 1
Hypochromic effects of **1** and **2** and their binding parameters with calf thymus DNA.[a]

Compound	H (%)[b]	K ($10^5\ M^{-1}$)	n	k_d (s^{-1})
1	50	0.45 ± 0.01	2	0.017
2	56	3.70 ± 0.93	2	0.760

[a] Experiments were conducted in 10 mM MES buffer (pH 6.25) and 100 mM NaCl at 25 °C.
[b] $H = 100\ (\varepsilon_f - \varepsilon_b)/\varepsilon_f$, where ε_f and ε_b refer to the molar absorptivity of free and DNA bound probe.

To study the dissociation processes of **1** and **2** from calf thymus DNA, kinetic measurements were made in 10 mM MES buffer (pH 6.25) and 100 mM NaCl at 30 °C. The dissocation rate constants for **1** and **2** from the complex with calf thymus DNA were found to be 0.017 and 0.76 s^{-1}, respectively (Table 1), demonstrating that the complex of **1** with double stranded DNA posseses 45-fold higher kinetic stability than that of **2**. These results demonstrate that **1** can stabilize kinetically its complex with double stranded DNA, but the binding constant of **1** was 8-times lower than that of **2**. This is reasonable in light of the fact that association is less favorable for **1**, as its bulky substituents need to pass through adjacent base pairs of double stranded DNA.

3.3. Optimization of effective masking reagent for gold substrate

Immobilization density of DNA on the gold surface is important to the hybridization efficiency [33,34]. However, the regarded surface is a bare gold, which can undergo non-specifc adsorption of oligonucleotides or **1**. To avoid such non-specific adsorption, alkanethiol having a hydroxyl group [35,36] and an oligoethylene group [37,38] have been developed as a self assembling monolayer (SAM) in this system. Although non-specific adsorption of oligonucleotides is known to avoid by the SAM of alkanethiols [35], it is not clear what kind of alkanethiol can avoid the non-specific adsorption of **1**. Thus we have tested masking properties of ME, MH, MU, and $MU(eg)_3$ using the gold substrate for **1** with preformed SAM by these reagents. FT-IR RAS measurement with **1** demonstrated that $MU(eg)_3$ masked the gold substrate most effectively (Fig. 6).

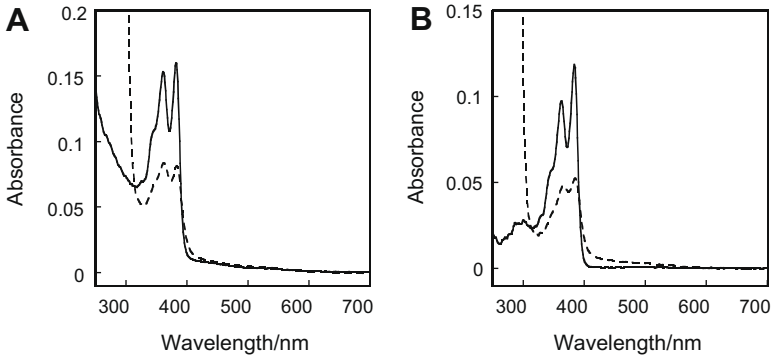

Fig. 3. Absorption spectra of 5 μM **1** (A) and **2** (B) in the absence (solid line) or presence of 2 mM (A) or 0.9 mM (B) calf thymus DNA (broken line) in 10 mM MES buffer (pH 6.25) and 100 mM NaCl at 25 °C.

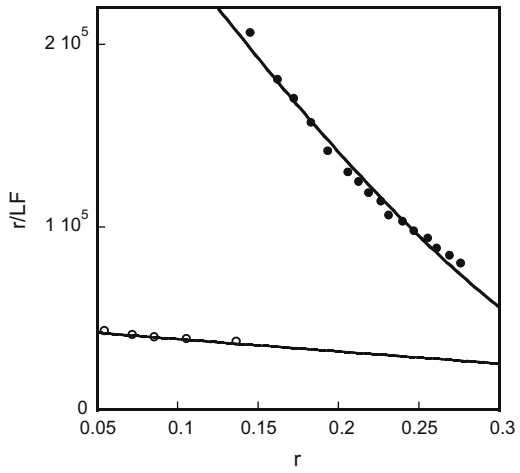

Fig. 4. Scatchard plots for **1** (○) or **2** (●) with calf thymus DNA in 10 mM MES buffer (pH 6.25) and 100 mM NaCl at 25 °C.

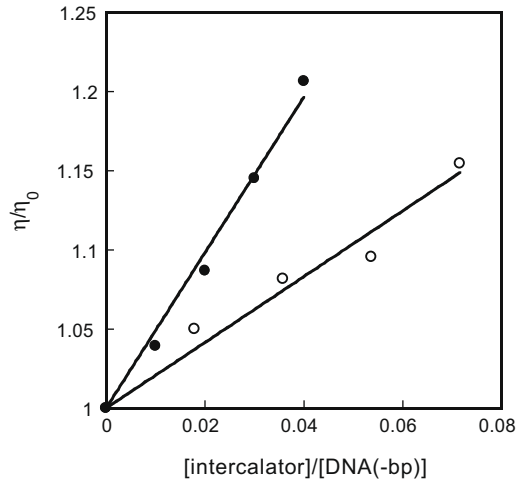

Fig. 5. Viscometric titration of 0.05 mM $[poly(dA\text{-}dT)]_2$ with a various amount of **1** (○) or **2** (●) at 30 °C.

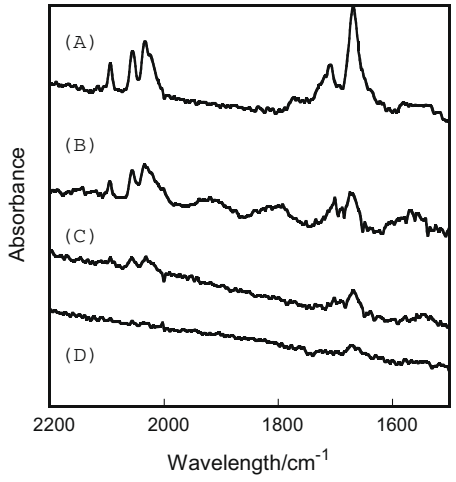

Fig. 6. FT-IR RAS spectra of the gold substrate masked with ME (A), MH (B), MU (C), or $MU(eg)_3$ (D) after treatment with **1** followed by washing with MilliQ water.

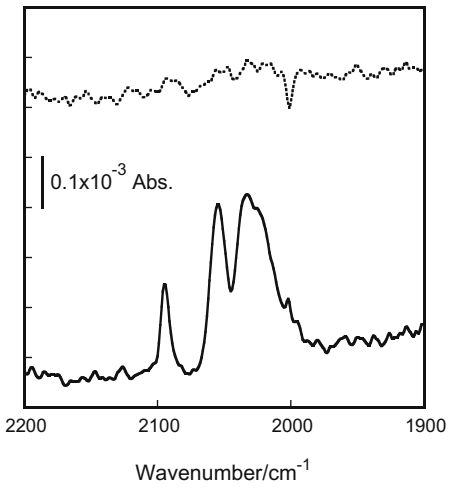

Fig. 7. FT-IR RAS spectra of an oligonucleotide-immobilized gold substrate before (broken line) and after hybridization with a complementary oligonucleotide (solid line).

*3.4. DNA hybridization detection by FT-IR RAS measurement in the presence of **1***

As a first step, the mixed SAM between thiolated oligonucleotide and $MU(eg)_3$ was prepared on the gold substrate. This could prepare by the treatment of the thiolated oligonucleotide in 2 × SSC for 18 h and subsequently treatment with $MU(eg)_3$ for 1 h at 25 °C. The formation of the mixed SAM was identified by the presence of the absorption assigned as nucleic bases of the oligonucleotide in FT-IR RAS measurement. However, absorption peaks around 2000 cm^{-1} based on the cobalt complex of **1** was not observed on the substrate after treatment with **1** and subsequently washing with water in the FT-IR RAS measurement (Fig. 7). This result shows that **1** could easily wash out on the single stranded DNA-immobilized gold substrate. However, the distinguishable absorption peaks based on **1** was observed in FT-IR RAS measurement after hybridization with the complementary oligonucleotide and subsequently treatment with **1** and water washing (Fig. 7). This showed that **1** can form stable complex with double stranded DNA on the substrate even after washing with water. The absorption peaks obtained here did not show any significant difference when compared with the peaks of Fig. 2. This suggested that the cobalt complex of **1** does not affect any local environmental change (free vs. intercalation).

4. Conclusion

Naphthalene diimide carrying dicobalt hexacarbonyl complexes **1** was synthesized and showed the strong binding with double stranded DNA by threading intercalation mode despite the presence of bulky substituent of the metal complexes. The bulky substituents are known to act as anchor to prevent dissociation of intercalator from double stranded DNA, when comparing with that for precursor **2**. Ability of **1** to serve as an IR probe for double stranded DNA was confirmed by the FT-IR RAS measurenet before and after hybridization with the complementary DNA on the DNA-immobilized gold surface after treatment with **1**. Since it is known that FT-IR ATR technique allowed DNA to measure in aqueous medium [39], **1** could use the monitoring the hybridization process of the target DNA with DNA probe in an aqueous medium, probably in a cell.

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