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Direct Labeling of 5-Methylcytosine and Its Applications

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Abstract: Cytosine methylation is one of the most important epigenetic events, and much effort has been directed to develop a simple reaction for methylcytosine detection. In this paper, we describe the design of tag-attachable ligands for direct methylcytosine labeling and their application to fluorescent and electrochemical assays. The effect of the location of bipyridine substituents on the efficiency of osmium complexation at methylcytosine was initially investigated. As a result, a bipyridine derivative with a substituent at the C4 position showed efficient complexation at the methylcytosine residue of single-stranded DNA in a reaction mixture containing potassium osmate and potassium hexacyanoferrate(III). On the basis of this result, a bipyridine derivative with a tag-attachable amino linker at the C4 position was synthesized. The efficiency of metal complex formation in the presence of the osmate and the synthetic ligand was clearly changed by the presence/absence of a methyl group at the C5 position of cytosine. The succinimidyl esters of functional labeling units were then attached to the bipyridine ligand fixed on the methylcytosine. These labels attached to methylcytosine enabled us to detect the target methylcytosine in DNA both fluorometrically and electrochemically. For example, we were able to fluorometrically obtain information on the methylation status at a specific site by means of fluorescence resonance energy transfer from a hybridized fluorescent DNA probe to a fluorescent label on methylcytosine. In addition, by the combination of electrochemically labeled methylcytosine and an electrode modified by probe DNAs, a methylcytosine-selective characteristic current signal was observed. This direct labeling of methylcytosine is a conceptually new methylation detection assay with many merits different from conventional assays.

Introduction

5-Methylcytosine is a common modified pyrimidine base, which frequently appears in genomic DNA, particularly in CpG sequences.[1] Cytosine methylation plays crucial roles in the regulation of chromatin stability, gene regulation, parental imprinting, and X chromosome inactivation.[2] In addition, the deactivation of the antioncogene caused by hypermethylation at the promoter region has been recognized as the cause of carcinogenesis.[3] Cytosine methylation is one of the most important epigenetic events, and its detection is very significant. Much effort has been directed to develop a simple reaction for methylcytosine detection.

For evaluation of the methylation status of genes, several conventional methods have so far been used, such as a cleavage assay with methylation-insensitive restriction enzymes,[4] and hydrolysis and sequencing with a bisulfite salt.[5] In the bisulfite assay, cytosines are converted into uracils via C4 hydrolysis and methylcytosines remain unchanged. Although the conventional methods have many merits, there are many disadvantages, and methylation detection assays must be further improved through another approach. The existence of a more rapid and selective chemical reaction capable of distinguishing between methylcytosine and nonmethylated cytosine would be promising as a good method for efficiently analyzing the status of cytosine methylation at a specific site in a gene. The five key points expected for a new chemical assay for methylation detection are as follows: (1) Methylcytosine-positive and cytosine-negative assay: Reactions selective to methylation sites should be developed. Unfortunately, conventional reactions are cytosine-selective but not methylcytosine-selective. (2) Sequence-

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selective assay: The focus of methylation studies is changing from the storage of information on the amount of methylation and the location of the methylation region to elucidation of the status and role of each methylation. Thus, the development of a conceptually new approach to site-specific detection is very important. (3) Fluorescent or electrochemical assay: The most facile method for methylation detection is probably to directly label the fluorescent or electrochemical signal-sending units to the methylation sites. There has never been a fluorescent or electrochemical assay for cytosine methylation detection to our knowledge, except for a DNA microarray of sequence-converted DNA via bisulfite treatment.[6] (4) Noncleavage assay: Most of the DNA sample is damaged by strand scission during the bisulfite treatment.[7] Nonspecific cleavage by drugs and enzymes complicates the detection process and lowers quantification precision. (5) Short-time assay: The bisulfite assay requires about a half day for complete modification to obtain reliable results. Shorter reaction time is desirable for the analysis of many samples.

The oxidation of pyrimidine bases may be applicable for the detection of the presence/absence of a methyl group at cytosine C5. The C5−C6 double bond of thymine and 5-methylcytosine bases is known to be oxidized by osmium tetroxide, and the bases are converted into their glycols.[8] Thymines in single-stranded DNAs are also oxidized by osmium tetroxide.[9] We have previously reported sequence-selective oxidation of methylcytosines.[10] We used the combination of potassium osmate, which is much less intractable than osmium tetroxide, potassium hexacyanoferrate(III) as an activator, and bipyridine as a reaction-accelerating ligand. Methylcytosines were oxidized efficiently by exposure to the reaction mixture, and a stable methylcytosine glycol−osmate−bipyridine complex was formed, making possible a clear distinction from inefficient oxidation of nonmethylated cytosine. In addition, methylcytosines in single-stranded DNA efficiently formed a metal complex, whereas the complexation of methylcytosines in a duplex was completely suppressed. The structural control of complexation efficiency was applicable to sequence-specific complexation.

Therefore, the oxidation of methylcytosine is promising for the detection of cytosine methylation status at a specific site of a long sequence, if the incorporation of tags into the methylcytosine in the DNA of interest would become possible through the coordination of functionalized bipyridine to the osmate. Using the attachment of a labelable amino group to the bipyridine ligand and complexation with methylcytosine, we can label methylcytosines with sequence-selective fluorescent or electrochemical units and analyze them methylcytosine-positively in a short time without any DNA scission (Figure 1). Thus, osmium complexation using a ligand with an amino linker would offer a promising prospect for a new methylation detection assay quite different from conventional ones.

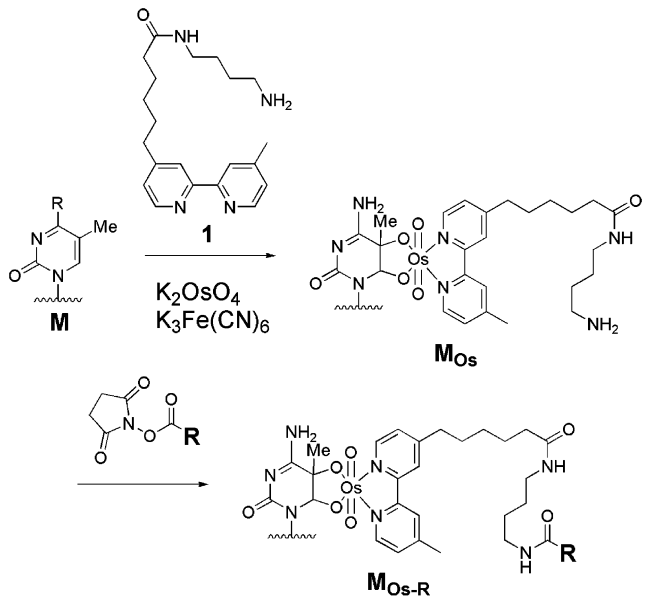

Figure 1. Schematic illustration of the labeling of methylcytosine via osmium complexation.

In this paper, we report the design of tag-attachable bipyridine ligands for direct methylcytosine labeling and their application to fluorescent and electrochemical assays. A functional bipyridine was prepared and incorporated into methylcytosine via complexation with osmate. Information on the methylation status at a specific site in the DNA was readily extracted by labeling the methylcytosine complex fluorometrically or electrochemically.

Results and Discussion

Design of Bipyridine Ligand with an Amino Linker. Toward the design of a bipyridine derivative with an amino linker, we initially investigated the effect of the position of the substituent of the bipyridine on osmium complexation, using two commercially available methyl-substituted bipyridines, 6-methyl-2,2′-bipyridine (**6sub**) and 4,4′-dimethyl-2,2′-bipyridine (**4sub**), and a ^{32}P-labeled oligodeoxyribonucleotide (ODN), 5′-^{32}P-d(AAAAAAGNGAAAAAA)-3′ (**ODN(N)**, N = 5-methylcytosine (M) or cytosine (C)). The bipyridines, dissolved in acetonitrile, were added to a mixture of potassium osmate, potassium hexacyanoferrate(III), and **ODN(N)** in Tris−HCl buffer (pH = 7.7), incubated at 0 °C for a period of 5 min, and then treated with hot piperidine to cleave the strands at the reaction sites for polyacrylamide gel electrophoresis analysis (Figure 2). Cleavage of **ODN(N)** by **6sub** was not observed (lanes 3 and 4), similar to the result for the reaction in the absence of ligand (lanes 9 and 10). On the other hand, for the reaction in the presence of **4sub**, **ODN(M)** was cleaved sequence-selectively at the methylcytosine site (lane 6), and strand cleavage at the cytosine of **ODN(C)** was completely suppressed (lane 5). The reactivity and selectivity in the presence of **4sub** were almost the same as those observed in the presence of a nonsubstituted bipyridine **Bpy** (lanes 1 and 2). The

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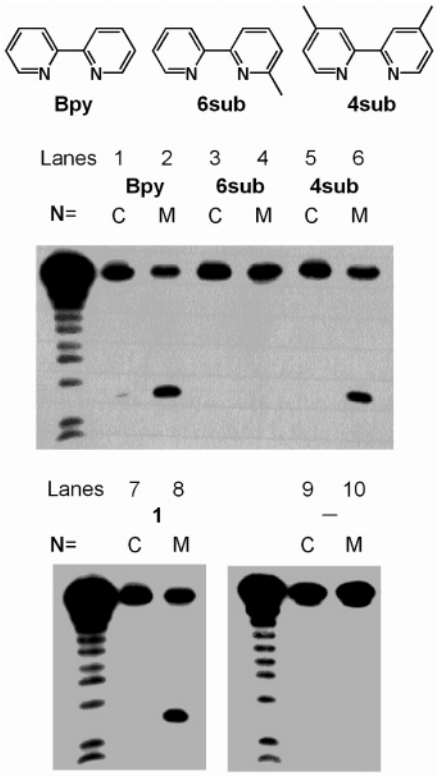


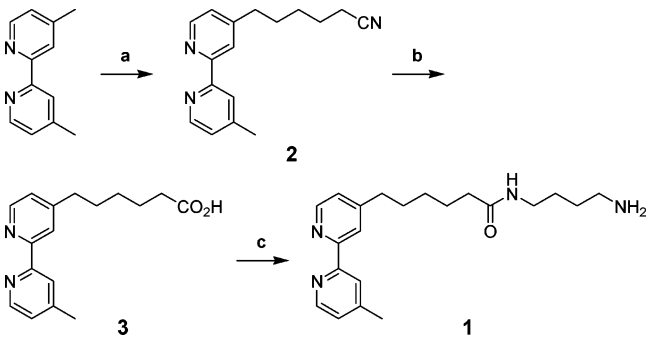
Figure 2. Osmium complexation at the methylcytosine site in the presence of bipyridine ligands. ^{32}P-labeled **ODN(N)**, 5′-^{32}P-d(AAAAAAGNGAAAAAA)-3′ (1 μM), was incubated in a solution of potassium osmate (5 mM), potassium hexacyanoferrate(III) (100 mM), ligand (100 mM), EDTA (1 mM) in Tris−HCl (100 mM, pH = 7.7), and 10% acetonitrile at 0 °C for a period of 5 min. The sample was then treated with hot piperidine (90 °C, 20 min). Lane 1, N = C in the presence of **Bpy**; lane 2, N = M in the presence of **Bpy**; lane 3, N = C in the presence of **6sub**; lane 4, N = M in the presence of **6sub**; lane 5, N = C in the presence of **4sub**; lane 6, N = M in the presence of **4sub**; lane 7, N = C in the presence of **1**; lane 8, N = M in the presence of **1**; lane 9, N = C, no ligand; Lane 10, N = M, no ligand. The ladder-like patterns are G+A sequencing lanes.

experimental results suggest that the site of the alkyl chain on bipyridine is significant for oxidation at methylcytosine. The substituent on bipyridine at C6 probably makes it sterically difficult to approach the methylcytosine in DNA, whereas **4sub** does not hinder the complexation with methylcytosine. Hence the incorporation of an amino linker to the bipyridine C4 position is suitable for methylation detection.

On the basis of the effective oxidation with **4sub**, we designed a bipyridine compound with an amino linker, 4-(6-(4-aminobutylamino)-6-oxohexyl)-4′-methyl-2,2′-bipyridine (**1**). The ligand **1** was synthesized from **4sub** (Scheme 1). The extension of the alkyl chain gave **2** (99%), and then acidic hydrolysis of the cyano group afforded **3** (97%). The connection of 1,4-diaminobutane to the carboxylic acid converted the alkyl chain terminus into an amino group to give **1** (85%).

We examined the osmium complexation of DNA using **1**. The strand cleavage observed after a 5 min reaction with osmate and **1** and then a hot piperidine treatment was negligible at the cytosine of **ODN(C)** (Lane 7), whereas **ODN(M)** was cleaved sequence-selectively at the methylcytosine site (Lane 8). The product given by the oxidation of **ODN(M)** was identified as a **1**−osmate−methylcytosine glycol triad, **ODN(M_{Os})** ([M − H]$^-$, calcd. 5233.85, found 5234.05). The complex was very stable in aqueous media, unless it was heated under alkaline conditions, such as the hot piperidine treatment described above.

Scheme 1 [a]



[a] Reagents and conditions: (a) (i) LDA, THF, −78 °C, 1 h, (ii) 5-bromovaleronitrile, 0 °C, 2 h, 99%; (b) concd HCl, 100 °C, 18 h, 97%; (c) diaminobutane, PyBOP, DMF, room temperature, 2 h, 85%.

The rate of metal complex formation was clearly controlled by the presence/absence of a methyl group at the cytosine C5 position. The half-life of 5′-d(GMG)-3′ (10 μM) under the complexation condition containing **1** was calculated as 11.0 min by HPLC monitoring. Although it was longer compared with that of 5′-d(GTG)-3′ (2.2 min), 5′-d(GMG)-3′ was completely consumed within 1 h. In contrast, 5′-d(GCG)-3′ decreased very slowly with a half-life of 365 min.

Incorporation of Labeling Agents at Complexation Sites. The formation of the methylcytosine-selective complex using a ligand with an amino linker made it possible to attach tags to the DNA methylation sites. The labeling reaction for the triad proceeded quantitatively using *N*-hydroxysuccinimidyl esters of the labels. Using this protocol, a variety of functional molecules, such as fluorophores, redox-active compounds, and bioaffinity compounds, were incorporated into **1** fixed on **ODN(M_{Os})** (Table 1). The fluorescein-labeled ODN, **ODN(M_{Os-F})** 5′-d(AAAAAAGM_{Os-F}GAAAAAA)-3′, which was derived from **ODN(M)** and fluorescein-6-carboxylic acid *N*-succinimidyl ester, showed a large fluorescence emission at 520 nm in sodium phosphate buffer (pH = 7.0) on excitation at 495 nm. In contrast, **ODN(C)** did not form a metal complex containing **1**, and thus the attachment of the fluorescence unit did not occur. The fluorescence labeling for methylcytosine using **1** enabled us to attach tags showing a positive signal for cytosine methylation of DNA.

Fluorescent Labeling for Sequence-Selective Methylcytosine Detection. Fluorescent labeling is one of the most powerful techniques for gene analysis and is frequently used for DNA sequencing, molecular beacons,[11] real-time PCR,[12] and DNA microarrays.[13] However, the direct fluorescence detection of methylcytosine has never been developed to our knowledge.

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Table 1. ODNs Modified with Osmium in This Study and Their MS Values[a]

	sequences	calcd	found
ODN(M_{Os})	5′-AAA AAA GM_{Os}G AAA AAA-3′	5233.85[b]	5234.05
ODN(M_{Os-HEX})	5′-AAA AAA GM_{Os-HEX}G AAA AAA-3′	5814.82[c]	5814.16
ODN(M_{Os}-BODIPY)	5′-AAA AAA G$M_{Os-BODIPY}$G AAA AAA-3′	5551.98[b]	5551.11
ODN(M_{Os}-F)	5′-AAA AAA GM_{Os-F}G AAA AAA-3′	5706.31[c]	5706.93
ODN($M_{Os-TAMRA}$)	5′-AAA AAA G$M_{Os-TAMRA}$G AAA AAA-3′	5647.30[c]	5647.97
ODN(M_{Os-AQ})	5′-AAA AAA GM_{Os-AQ}G AAA AAA-3′	5468.06[b]	5467.46
ODN($M_{Os-biotin}$)	5′-AAA AAA G$M_{Os-biotin}$G AAA AAA-3′	5476.14[b]	5475.71

[a] Mass spectra were taken by MALDI-TOF MS with THAP as a matrix. [b] The values were calculated as $[M - H]^-$. [c] The values were calculated as $[M - OH]^-$.

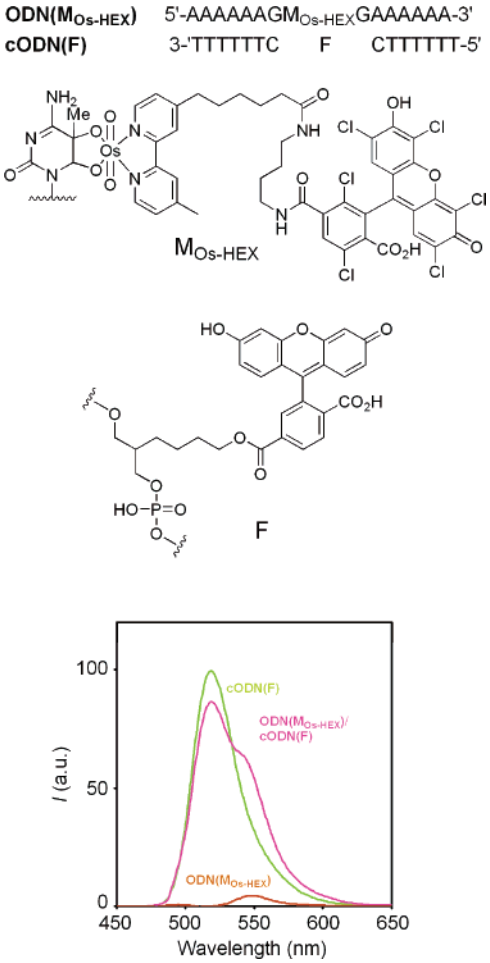

Figure 3. Fluorescence spectra of fluorescent labeled duplexes. 1 μM **ODN(M_{Os-HEX})** (orange line), 1 μM **cODN(F)** (green line), and a 1 μM solution of the duplex of **ODN(M_{Os-HEX})** and **cODN(F)** (magenta line) were measured in 50 mM sodium phosphate (pH = 7.0) and 0.1 M sodium chloride at 25 °C. Excitation was at 495 nm.

Fluorescent labeling mediated by the complexation of methylcytosine, osmate, and **1** would be applicable to the direct detection of cytosine methylation status at a desired sequence. In particular, by applying a fluorescence resonance energy transfer (FRET) system, in which the efficiency strongly depends on the interchromophore distance, the methylation status of the target cytosine could be estimated efficiently, fluorometrically discriminating the target methylation site from thymines and other methylcytosines. On the basis of this concept, we applied the fluorophore incorporated onto a methylcytosine site through an osmium−**1** complex to a FRET system from the neighboring fluorophores fixed to the hybridizing DNA. We prepared **ODN(M_{Os-HEX})**, 5′-d(AAAAAAGM_{Os-HEX}GAAAAAA)-3′, which was given by the methylcytosine-selective labeling of **ODN(M)** with hexachlorofluorescein (HEX), and investigated the FRET efficiency from the complementary ODN containing a fluorescein unit, **cODN(F)** 5′-d(TTTTTTCFCTTTTTT)-3′. In the fluorescence spectrum of the duplex on excitation at 495 nm, in which the fluorescein is excited selectively, a signal at 535 nm was observed as a shoulder of a signal at 520 nm, which originates from the fluorescein of **cODN(F)** (Figure 3). The shoulder signal was from HEX, and its intensity was much larger than that of HEX fluorescence on excitation at 495 nm, suggesting that FRET occurred from **cODN(F)** to **ODN(M_{Os-HEX})**. The FRET efficiency from fluorescein to HEX in this system was calculated to be 43%. The fluorescence intensity of the duplex at 535 nm was 30 times that of the single-stranded **ODN(M_{Os-HEX})**.

The cytosine residue of codon 175 in exon 5 of the p53 gene is known as a mutation hotspot, which is significantly connected with carcinogenesis.[14] We treated the 60-mer p53 fragment containing this methylation hotspot, 5′-d(...GGCAGNGCCTCA...)-3′ (**p53(N)**, N = C or M), with the osmate and **1**, and then added the *N*-hydroxysuccinimidyl ester of HEX to the reaction solution (Figure 4). The reaction mixture was desalted through a gel filter, and then the recovered DNA was added to a solution containing a fluorescein-labeled probe, **cODN$_{p53}$(F)**. The fluorescence intensity of each sample at 535 nm on excitation at 495 nm was compared with that of the control sample. A slight difference in fluorescence intensity was observed for **p53(C)**. On the other hand, the fluorescence from **p53(M)** increased. In addition, it is noteworthy that cytosine methylation at the desired site was analyzed with a high sequence selectivity, although there are many labelable thymines in the sequence. The results clearly show that FRET worked effectively only for the HEX label of the methylcytosine, which was located in the immediate neighborhood of the fluorescein of **cODN$_{p53}$(F)**. Direct fluorescent labeling to methylcytosine enabled us to visually judge the presence/absence of methylation of a cytosine, and the sequence-selective analysis of cytosine methylation also became possible by the use of FRET.

Electrochemical Labeling for Methylcytosine Detection. Electrochemical analysis is also an efficient technique that extracts only the information required from a large amount of gene information. Electrochemical DNA biosensors can be widely applied, for example, to single-nucleotide polymorphism detection,[15] the evaluation of anticancer drugs,[16] tracing interac-

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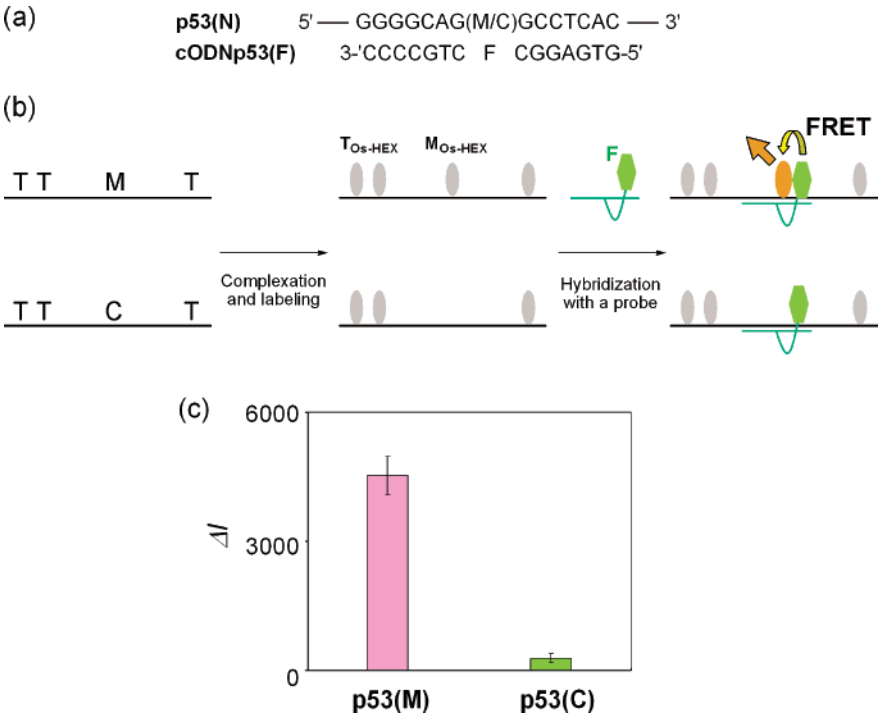

Figure 4. Sequence-selective methylcytosine sensing using FRET. (a) Target sequences containing a methylation hotspot in the p53 gene, **p53(N)**, and a probe DNA involving a fluorescein linker, **cODN$_{p53}$(F)**. (b) Schematic procedure for the sequence-selective sensing of cytosine methylation. (c) Changes in the fluorescence intensity of **p53(N)** target DNAs at 535 nm. The fluorescence of labeled **p53(N)** was measured in the presence of **cODN$_{p53}$(F)** (1 μM) in sodium phosphate (50 mM, pH = 7.0) and sodium chloride (0.1 M) at 25 °C. A 495 ± 7 nm band-pass filter and a 535 ± 12.5 nm band-pass filter were used for excitation and emission, respectively. ΔI is the value given by subtracting the fluorescence intensity observed for a reaction solution that did not contain **p53(N)** target DNAs from the fluorescence intensity of the reaction sample. The data points represent the average of five experimental runs and the 10% error bars are retained from the individual data sets.

tions with biomolecules,[17] and microassay for small molecules.[18] Although the analysis of cytosine methylation using an electrochemical assay has never been reported, the formation of the **1**−osmate−methylcytosine glycol triad would facilitate labeling with electroactive units to help electrochemical detection.

For an electrochemical assay, we designed a thiolated ODN (**cODN-S** 5′-d(TTTTTTCCTTTTTT)-SH-3′) as the target strand capture DNA, immobilized on gold electrodes using the sulfur−gold interaction. Self-assembled monolayer surfaces of **cODN-S** were prepared by immersing a gold electrode (2 mm^2 in area) in a solution of a thiolated DNA. By hybridization with **ODN-(N)**, the bulged duplex was assembled on the surface of the gold electrode ((1.62 ± 0.36) × 10^{13} DNA cm^{-2}).[19] Square wave voltammetry (SWV) measurements were carried out in 50 mM sodium phosphate buffer (pH = 7.0) and 0.1 M sodium chloride. The current responses of the duplexes, using a saturated calomel electrode (SCE) as a reference electrode, are shown in Figure 5. Nonmodified **ODN(M)** showed no current signal in the voltage range between 0 to −0.8 V, where it is known that the DNA film on the gold electrode is relatively stable. The electrode modified by **ODN(M$_{Os}$)**, obtained after osmium complexation, exhibited a small current signal at approximately −0.8 V. On the other hand, **ODN(M$_{Os-AQ}$)**, labeled with anthraquinonecarboxylic acid, gave a large current peak at −0.7 V, assigned as the redox potential of anthraquinonecarboxamide. Although **ODN(C)** treated with osmate, **1**, and anthraquinonecarboxylic acid *N*-succinimidyl ester was also hybridized with **cODN-S** on an electrode, the reaction sample showed no current signal. These current signal intensities suggest that a methylcytosine in DNA can be labeled with the anthraquinone through complex formation with osmate and **1**, and methylcytosines can be detected as an electrochemical signal.

AC impedance measurement is well-known as an effective electrochemical assay technique. An assay system to determine the methylation status at a specific cytosine residue in p53 DNA with impedance measurement was examined. We designed the electrochemical protocol as shown in Figure 6. A thiolated probe DNA for p53 DNA analysis, **cODN$_{p53}$-S**, was placed on a gold electrode. The single base bulge at the target cytosine residue was formed by hybridization of the target **p53(N)** DNA (N = C or M)[14] with **cODN$_{p53}$-S** on the electrode. A fully matched complementary ODN, **pODN$_{p53}$**, was exposed to the electrode to prevent osmium complexation of nonhybridized **cODN$_{p53}$-S**. Complex formation with the osmate in the presence of **1** was then performed according to the conditions described above. Subsequently, S1 nuclease treatment was executed to delete the undesired complexation caused in the single-stranded region of the target DNA. Biotin was attached to the immobilized DNA via **1**−osmate complex formation, and then an avidin−horseradish peroxidase (HRP) conjugate was fixed on the biotin unit. The impedance behavior was then examined for the electrodes covered with the precipitate of 4-chloro-1-naphthol oxidized by HRP.

The impedance features of the assemblies, using $[Fe(CN)_6]^{3-/4-}$ as the redox probe, are presented as Nyquist plots (Figure 7).

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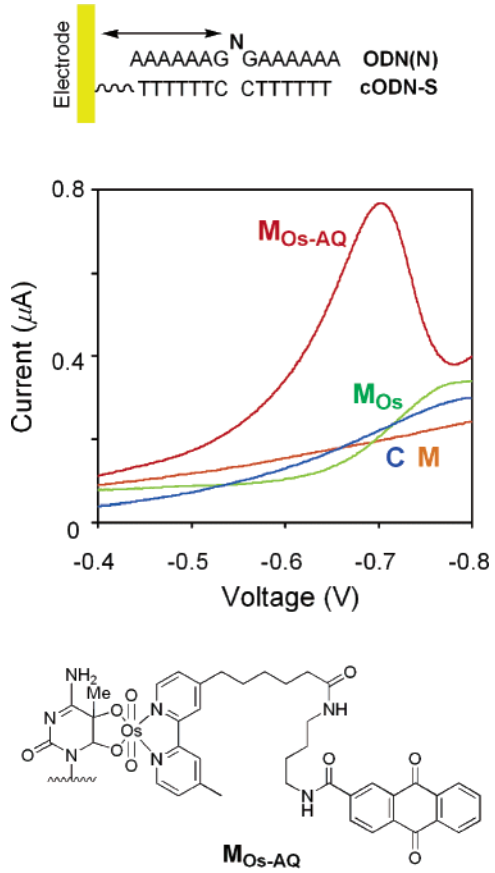

Figure 5. Square wave voltammetry (SWV) for electrodes modified by **ODN(N)/cODN-S**. The electrochemical signal of **ODN(M)**, **ODN(M_{Os})**, and **ODN(M_{Os-AQ})** are shown as the orange, green, and red lines, respectively. **ODN(C)** was also hybridized with **cODN-S** on an electrode after a series of treatments including oxidation and labeling under the same conditions as the **ODN(M_{Os-AQ})** preparation (blue line). SWV was measured in 1 M sodium chloride with the DNA modified electrode, with a SCE for the reference electrode and Pt for the counter electrode. Pulse amplitude 10 mV; pulse width 50 ms; frequency 15 Hz.

The interfacial electron-transfer resistance[20] R_{et} of the assembly, given after the series of treatments for **p53(C)** (curve b, R_{et} = 17.7 kΩ), was almost same as the value observed for an electrode not hybridized with **p53(N)** (curve a, R_{et} = 18.0 kΩ), whereas the assembly derived from **p53(M)** showed a higher resistance (curve c, R_{et} = 26.8 kΩ). The **p53(M)** electrode showed a higher impedance than those of the **p53(C)** electrode and nontarget electrode, and **p53(M)** was electrochemically discriminated from **p53(C)**. S1 nuclease treatment is very significant for deleting the background signal. Without S1 nuclease treatment, the R_{et} values of both methylated and nonmethylated samples were high and showed slight differences (R_{et} = 37.4 kΩ for **p53(M)**, 33.7 kΩ for **p53(C)**).

In conclusion, we have described the design of tag-attachable bipyridine ligands for direct methylcytosine labeling and their application to fluorescent and electrochemical assays. A bipyridine with a tag-attachable amino linker, **1**, was designed and incorporated into methylcytosine via complexation with osmate. Incorporation of a variety of functional units into the methylcytosine in the DNA of interest became possible by coordination of **1** to the osmate. This complexation fluorometrically and electrochemically distinguished 5-methylcytosine from nonmethylated cytosine located in a specific sequence. Direct methylcytosine labeling with such functional units has been undertaken for the first time, and makes many approaches to effective methylcytosine detection possible. Direct methylcytosine labeling is a quite a conceptually new method for methylation detection, but several points still remain to be improved for effective epigenotyping. One is that osmium complexation occurs for thymines as well as methylcytosine in single-stranded DNA. The reaction at thymines competing with methylcytosines complicates the sequence-selective methylcytosine detection process. As described in text, the use of steric control, such as bulge structure formation, and distance-dependent control such as FRET may be effective for sequence-selective cytosine methylation sensing. Another issue is signal sensitivity. Although osmium complexation clearly distinguishes methylcytosine from nonmethylated cytosine and helps to give a methylation-selective signal, the signal intensity and sensitivity may be a bit weak in practical experiments using a small amount of genome sample. Therefore, the development of amplification of the given signal is important for highly sensitive epigenotyping. However, the present methylcytosine labeling approach includes many advantages quite different from conventional assays. Using incorporation of a tag-attachable amino group to a bipyridine ligand and complexation with methylcytosine, we can label methylcytosines with fluorescent or electrochemical units and analyze them sequence-selectively and methylcytosine-positively in a short time without any DNA scission. The direct labeling of methylcytosine solves all of the problems of the conventional assays raised in the introduction section of this text. Thus, osmium complexation using a ligand with an amino linker offers a promising prospect for a new methylation assay quite different from conventional ones. Further improvement of this methylcytosine labeling will afford a more reliable epigenotyping method, taking the place of conventional methylation analysis.

Experimental Section

General. ^{1}H NMR spectra were measured with Varian Mercury 400 (400 MHz) spectrometer. ^{13}C NMR spectra were measured with JEOL JNM α-500 (125 MHz) spectrometer. Coupling constants (*J* value) are reported in hertz. The chemical shifts are expressed in ppm downfield from tetramethylsilane, using residual chloroform (δ = 7.24 in ^{1}H NMR, δ = 77.0 in ^{13}C NMR) as an internal standard. FAB mass spectra were recorded on JEOL JMS DX-300 spectrometer or JEOL JMS SX-102A spectrometer. Masses of ODNs were determined with a MALDI-TOF mass spectroscopy (acceleration voltage 21 kV, negative mode) with 2′,3′,4′-trihydroxyacetophenone (THAP) as matrix, using T_8 ([M − H]$^-$ 2370.61) and T_{17} ([M − H]$^-$ 5108.37) as an internal standard.

4-(5-Cyanopentyl)-4′-methyl-2,2′-bipyridine (2). To a solution of diisopropylamine (4.6 mL, 33 mmol) in THF (100 mL) was added *n*-butyllithium (1.6 M solution in hexane, 18.4 mL, 29.4 mmol), and the reaction mixture was stirred at −78 °C for 15 min. After addition of 4,4′-dimethyl-2,2′-bipyridine (5.16 g, 28 mmol) in THF (100 mL), the reaction mixture was strirred for 1 h at −78 °C. To the solution was added 5-bromovaleronitrile (3.6 mL, 31 mmol) in THF (5 mL), and the reaction mixture was stirred for 2 h at 0 °C. The resulting mixture was diluted with water (100 mL), neutralized with 1 N HCl, and extracted with ethyl acetate. The organic phase was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (hexane−ethyl acetate = 10:1) to yield **2** (7.40 g, 27.9 mmol, 99%) as a brown powder; ^{1}H NMR ($CDCl_3$) δ 8.51 (dd, 1H, *J* = 0.7, 5.1 Hz), 8.49 (dd, 1H, *J* =

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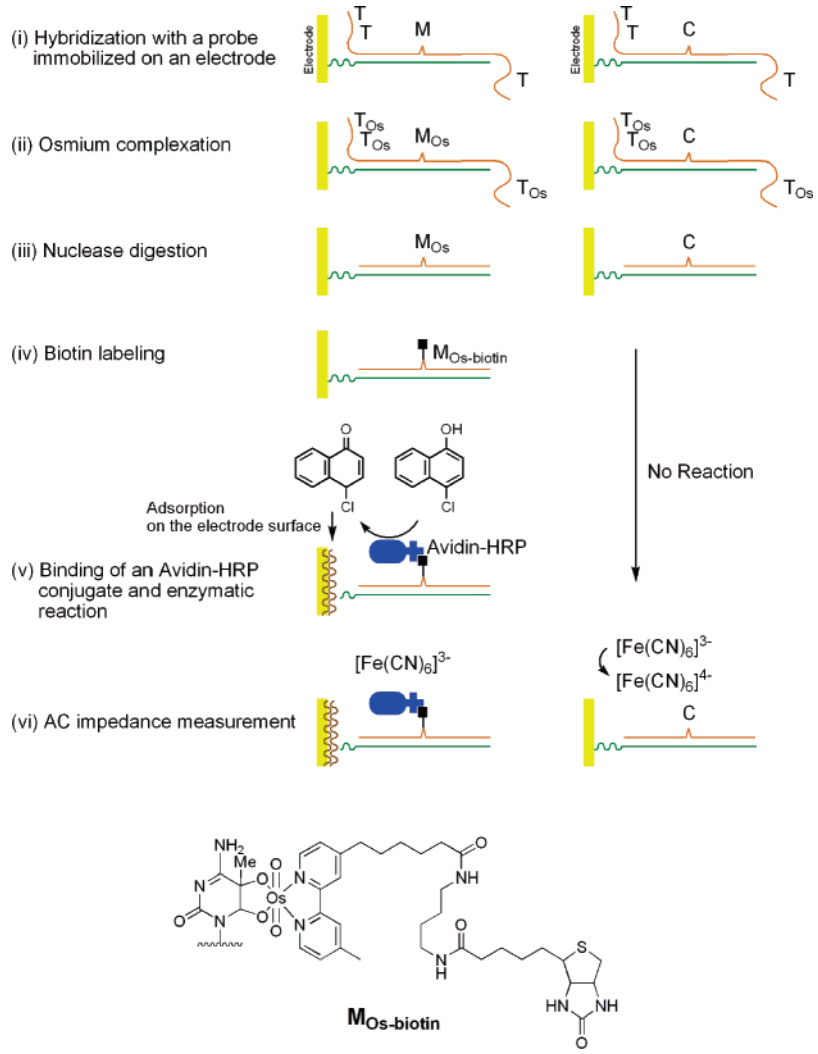

Figure 6. Schematic procedure for osmium modification and electrochemical measurement of DNA-modified gold electrodes. (i) A target DNA is hybridized with a probe immobilized on a gold electrode. A single-base bulge is formed at a target site. (ii) The target DNA on the electrode is treated with osmate and **1** after nonhybridized probes on the electrode are protected. (iii) The single-stranded region is digested with S1 nuclease to lower background errors. (iv) Biotin is coupled with an amino group at **1**. (v) An avidin−HRP conjugate is attached to biotin on methylcytosine. 4-Chloronaphthol is added and converted into a hydrophobic product, which adsorbs to an electrode surface, by an avidin−HRP conjugate. (vi) In the AC impedance measurement, the complex from methylcytosine shows a high resistance.

0.5, 4.9 Hz), 8.19−8.18 (m, 2H), 7.09−7.06 (m, 2H), 2.66 (t, 2H, J = 7.7 Hz), 2.33 (s, 3H), 2.28 (t, 2H, J = 7.0 Hz), 1.73−1.60 (m, 4H), 1.50−1.44 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 155.9, 155.6, 151.6, 148.8, 148.6, 147.8, 124.4, 123.5, 121.7, 120.8, 119.3, 34.8, 29.2, 27.9, 24.9, 20.8, 16.7; FABMS (NBA/$CHCl_3$) m/z 266 ([(M + H)$^+$]), HRMS calcd. for $C_{17}H_{20}ON_3$ ([(M + H)$^+$]) 266.1657, found 266.1657.

6-(4′-Methyl-2,2′-bipyridin-4-yl)hexanoic Acid (3). A mixture of **2** (7.40 g, 27.9 mmol) and concentrated HCl (50 mL) was stirred at 100 °C overnight. The resulting mixture was adjusted the pH value to 4.0−4.5 with 6 N NaOH, and extracted with chloroform. The organic phase was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo* to yield **3** (7.70 g, 27.1 mmol, 97%) as a magenta powder; ^{1}H NMR ($CDCl_3$) δ 8.57 (d, 1H, J = 0.7 Hz), 8.56 (d, 1H, J = 0.7 Hz), 8.21 (s, 1H), 8.18 (d, 1H, J = 0.7 Hz), 7.17 (ddd, 1H, J = 0.7, 1.6, 5.1 Hz), 7.14 (dd, 1H, J = 1.8, 5.1 Hz), 2.72 (t, 2H, J = 7.7 Hz), 2.45 (s, 3H), 2.36 (t, 2H, J = 7.3 Hz), 1.77−1.67 (m, 4H), 1.46−1.40 (m, 2H); ^{13}C NMR ($CDCl_3$) 177.7, 155.5, 152.8, 148.7, 148.6, 148.5, 124.7, 124.0, 122.6, 121.8, 35.1, 34.0, 29.7, 28.5, 24.5, 21.1; FABMS (NBA/$CHCl_3$) m/z 285 ([(M + H)$^+$]), HRMS calcd. for $C_{17}H_{21}O_2N_2$ ([(M + H)$^+$]) 285.1603, found 285.1612.

***N*-(4-Aminobutyl)-4-methyl-4′-(2,2′-bipyridyl)hexanamide (1).** A mixture of **3** (568 mg, 2.0 mmol) and PyBOP (1.14 g, 2.2 mmol) in DMF (10 mL) was stirred at room temperature for 30 min. To the solution was added 1,4-diaminobutane (222 μL, 2.2 mmol), and the mixture was stirred at room temperature for 2 h. The resulting mixture was concentrated and then diluted with chloroform. The organic phase was washed with 1 N NaOH and brine, dried over $MgSO_4$, and concentrated *in vacuo* to yield **1** (603 mg, 1.7 mmol, 85%) as a brown oil; ^{1}H NMR ($CDCl_3$) δ 8.45 (t, 2H, J = 5.4 Hz), 8.12 (d, 2H, J = 6.8 Hz), 7.05−7.02 (m, 2H), 6.71 (t, 1H, J = 5.4 Hz), 3.15−3.70 (m, 4H), 2.60 (t, 2H, J = 7.8 Hz), 2.35 (s, 3H), 2.09 (t, 2H, J = 7.3 Hz), 1.66−1.55 (m, 4H), 1.42−1.39 (m, 4H), 1.32−1.25 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 173.1, 155.9, 152.4, 148.8, 148.7, 148.0, 124.5, 123.8, 121.9, 121.1, 38.8, 36.2, 35.1, 29.9, 28.7, 26.7, 25.3, 20.1; FABMS (NBA/$CHCl_3$) m/z 355 ([(M + H)$^+$]), HRMS calcd. for $C_{21}H_{31}ON_4$ ([(M + H)$^+$]) 355.2498, found 355.2498.

ODN Synthesis and Characterization. ODNs were synthesized by the conventional phosphoramidite method by using an Applied Biosystems 392 DNA/RNA synthesizer. Synthesized ODNs were purified by reverse phase HPLC on a 5-ODS-H column (10 × 150 mm, elution with a solvent mixture of 0.1 M triethylammonium acetate (TEAA), pH 7.0, linear gradient over 30 min from 5% to 20% acetonitrile at a flow rate 3.0 mL/min). An aliquot of purified ODN solution was fully digested with calf intestine alkaline phosphatase (50 U/mL), snake

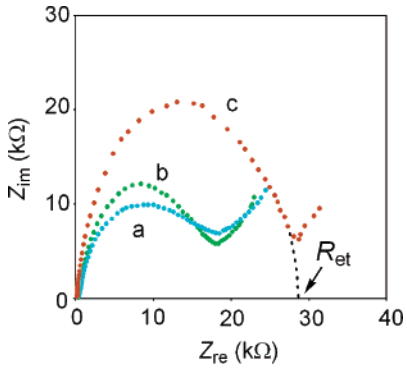

Figure 7. Nyquist diagram (Z_{im} *vs* Z_{re}) for Faradaic impedance measurements of **p53(N)**. The target DNA **p53(N)** was treated according to the protocol shown in Figure 6. (a) An electrode not hybridized with **p53(N)**; (b) the assembly derived from **p53(C)**; (c) the assembly derived from **p53-(M)**. All measurements were recorded in a phosphate buffer solution (50 mM, pH = 7.0) using $[Fe(CN)_6]^{3-/4-}$ (10 mM), as the redox probe. The impedance spectra were recorded within the frequency range 0.1 Hz to 10 kHz at the formal potential of the $[Fe(CN)_6]^{3-}$ redox couple. The amplitude of the alternating voltage was 5 mV.

venom phosphodiesterase (0.15 U/mL), and P1 nuclease (50 U/mL) at 37 °C for 3 h. Digested solution was analyzed by HPLC on Cosmosil 5C-18AR or CHEMCOBOND 5-ODS-H column (4.6 × 150 mm), elution with a solvent mixture of 0.1 M triethylammonium acetate (TEAA), pH 7.0, linear gradient over 20 min from 0% to 20% acetonitrile at a flow rate 1.0 mL/min). Concentration of each ODN was determined by comparing a given peak area with those of 0.1 mM standard solution containing dA, dC, dG, and dT. Each ODN was characterized by MALDI-TOF MS. **ODN(M)**, 5′-d(AAAAAAGM-GAAAAAA)-3′ ($[M - H]^-$, calcd 4657.13, found 4657.14); **cODN-(F)**, 5′-d(TTTTTTCFCTTTTTT)-3′ ($[M - H]^-$, calcd. 4733.20, found 4733.42); **cODN$_{p53}$(F)**, 5′-d(GTGAGGCFCTGCCCC)-3′ ($[M - H]^-$, calcd. 4809.25, found 4809.62); **cODN-S**, 5′-d(TTTTTTCCTTTTTT)-SH-3′ ($[M - H]^-$, calcd. 4361.92, found 4361.81); **cODN$_{p53}$-S**, 5′-d(GTGAGGCCTGCCCC)-SH-3′ ($[M - H]^-$, calcd. 4435.94, found 4435.29). The mass values of ODNs forming an osmium complex are summarized in Table 1.

Preparation and Osmium Oxidation of ^{32}P-5′-End-Labeled Oligodeoxynucleotides. The ODNs (400 pmol-strand) were 5′-end-labeled by phosphorylation with 4 μL of [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase using a standard procedure. The 5′-end-labeled ODN was recovered by ethanol precipitation and further purified by 15% denaturing polyacrylamide gel electrophoresis (PAGE). The ODN to be examined was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM the synthetic ligand, **1**, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7), and 10% acetonitrile at 0 °C for 5 min. A reaction mixture was ethanol-precipitated with the addition of 15 μL of 3 M sodium acetate (pH = 5.0), 10 μL of salmon sperm DNA (1 mg/mL), and 1 mL of cold ethanol. The precipitated ODN was washed with 150 μL of 80% cold ethanol and dried *in vacuo*. The precipitated ODN was resolved in 50 μL of 10% piperidine (*v*/*v*), heated at 90 °C for 20 min, evaporated by vacuum rotary evaporation to dryness, and then resuspended in 5−20 μL of 80% formamide loading buffer (a solution of 80% formamide (*v*/*v*), 1 mM EDTA, 0.1% xylenecyanol, and 0.1% bromophenol blue). The samples (1 μL, 3−10 kcpm) were loaded onto 15% denatureing 19:1 acrylamide−bisacrylamide gel containing 7 M urea, electrophoresed at 1900 V for approximately 1 h, and transferred to a cassette and stored at −80 °C with X-ray film.

Osmium Oxidation and Labeling of Oligodeoxynucleotides. The ODN (5 μM) to be examined was incubated in a solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM the synthetic ligand, **1**, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7), and 10% acetonitrile at 37 °C for 3 h. Purification and characterization of ODNs after osmium complexation were archived with the same methods as ODN synthesis. *N*-Hydroxysuccinimide esters of probe molecules (50 mM) in DMF (10 μL) was added to a 5 μM solution (total volume 100 μL) of osmated DNA in 50 mM sodium phosphate (pH = 8.0) and incubated at room temperature for 3 h. The reaction mixture was purified by HPLC with the same condition as above.

Fluorescence Measurements. All fluorescence spectra of the ODNs (1 μM, final duplex concentration) were taken in 50 mM sodium phosphate buffers (pH 7.0) containing 100 mM sodium chloride. Fluorescence spectra were obtained using a Shimadzu RF-5300PC spectrofluorophotometer at 25 °C using 1 cm path length cell. The excitation bandwidth was 1.5 nm. The emission bandwidth was 1.5 nm. The FRET efficiency was determined according to the reference, *Biophys. J.* **2004**, *86*, 371−383.

Discrimination between Cytosine and Methylcytosine at the p53 Hotspot. Single-stranded **p53(C)** or **p53(M)** (10 μM) was incubated in 100 μL of the reaction solutions containing 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM bipyridine, 1 mM EDTA in 100 mM Tris-HCl buffer (pH = 7.7), and 10% acetonitrile at 37 °C for 1 h, followed by adding 10 μL of 1 mg/mL biotin-NHS solution in DMSO. Each solution was desalted by Micro Bio-Spin Chromatography Columns. After drying *in vacuo*, the solutions for fluorescence analysis containing 1 μM of **cODN$_{p53}$(F)** in 50 mM sodium phosphate buffers (pH 7.0) containing 100 mM sodium chloride were prepared. Fluorescence analysis on a microtiter plate was performed. The fluorescence was measured through a 495 ± 7 nm band-pass excitation filter and a 535 ± 12.5 nm band-pass emission filter. The count time was 0.1 s, and the lamp energy was 7000.

Preparation of Modified Gold Electrodes for SWV Measurements. A gold electrode with an area of 2 mm^2 was used for this study. Prior to DNA immobilization, it was soaked in boiling 2 M potassium hydroxide for 3 h and washed with deionized water. The electrode was then soaked in concentrated nitric acid for 1 h and washed with deionized water. For chemisoption of DNA, a 1-μL solution of 10 μM thiolated probe DNA was placed on a gold electrode held upside-down, and the end of the electrode was fitted with a rubber cap to protect the solution from evaporation. The assembly was kept standing for 2 h at room temperature. Subsequently, a 1-μL solution of 10 μM anthraquinone-labeled ODN was placed on a gold electrode held upside-down, and the end of the electrode was fitted with a rubber cap to protect the solution from evaporation. The assembly was kept standing for 30 min at room temperature.

SWV Measurements. The current was measured in a three-electrode arrangement (ALS, model 660A), a modified Au working electrode (electrode area, 2 mm^2), a platinum counter electrode, and a SCE reference electrode at 25 °C. The current measurements were performed in 50 mM sodium phosphate (pH = 7.0) and 0.1 M sodium chloride. SWV conditions were 5 mV step height, 25 mV of amplitude, 1 s quiescent time, initial potential 0 V, end potential −1.5 V, and 15 Hz frequency.

Determination of Surface Density of Probe DNA on a Gold Electrode. The amount of DNA immobilized on the electrode was determined using a chronocoulometric assay in the presence of ruthenium(III) hexaammine, according to Tarlov's method.[19]

Discrimination between Cytosine and Methylcyotosine at the Methylation Hot Spot with the Faradaic Impedance Measurement. A gold electrode with an area of 2 mm^2 was used for this study. Prior to DNA immobilization, it was soaked in boiling 2 M potassium hydroxide for 3 h and washed with deionized water. The electrode was then soaked in concentrated nitric acid for 1 h and washed with deionized water. For chemisoption of DNA, a 1-μL solution of 10 μM thiolated probe DNA, **cODN$_{p53}$-S** 5′-d(GTGAGGCCTGCCCC)-SH-3′, was placed on a gold electrode held upside-down, and the electrode was kept standing for 15 min at room temperature. After washing with deionized water, a 1-μL solution of 1 μM target DNA, **p53(N)** (N =

M or C) 5′-d(GCTATCTGAGCAGCGCTCATGGTGGGGGCAGN-GCCTCACAACCTCC GTCATGTGCTGTGA)-3′, was placed on a gold electrode held upside-down, and the electrode was kept standing for 5 min at room temperature. After washing with 1 M sodium chloride, for prohibiting of nonspecific osmation on the probe DNA, a 1-μL solution of 10 μM the protection ODN, **pODN$_{p53}$** 5′-d(GGGGCAG-GCCTCAC)-3′, was placed on a gold electrode held upside-down, and the electrode was kept standing for 5 min at room temperature. After washing with 1 M sodium chloride, a 1-μL solution of 5 mM potassium osmate, 100 mM potassium hexacyanoferrate(III), 100 mM solution of the synthetic ligand, **1**, 1 mM EDTA in 100 mM Tris−HCl buffer (pH = 7.7), and 10% acetonitrile was placed on a gold electrode held upside-down, and the end of the electrode was fitted with a rubber cap to protect the solution from evaporation. The assembly was kept standing for 45 min at room temperature. After washing with 1 M sodium chloride, a 1-μL solution of 0.5 U S1 endonuclease in 30 mM sodium acetate (pH = 4.6), 100 mM sodium chloride, and 1 mM $ZnSO_4$ was placed on a gold electrode held upside-down, and the electrode was kept standing for 5 min at room temperature. The reaction was terminated by adding a 1-μL solution of 10 mM EDTA and 1 M Tris-HCl buffer (pH = 7.7) on a gold electrode held upside-down. Subsequently, a 1-μL solution of 1 M sodium chloride, 10% DMSO, and 50 mM *N*-hydroxysuccinimidyl biotin ester was placed on a gold electrode held upside-down, and the end of the electrode was fitted with a rubber cap to protect the solution from evaporation. The assembly was kept standing for 45 min at room temperature. After washing with 1 M sodium chloride, a 1-μL solution of avidin−HRP conjugate solution (BioRad) was placed on a gold electrode held upside-down, and the electrode was kept standing for 5 min at room temperature. After washing with 1 M sodium chloride, for a precipitation reaction, a 1-μL solution of 1 mM 3-chloro-1-naphthol, 0.5% hydroxyl peroxide, and 100 mM sodium phosphate buffer (pH = 7.0) was placed on a gold electrode held upside-down, and the electrode was kept standing for 5 min at room temperature. After washing with deionized water, Faradaic impedance measurements were performed.

Faradaic Impedance Measurements. The Faradaic measurement was performed in a three-electrode arrangement (ALS, model 660A), a modified Au working electrode (electrode area, 2 mm^2), a platinum counter electrode, and a SCE reference electrode in a phosphate buffer solution, 50 mM, pH = 7.0, using $[Fe(CN)_6]^{3-/4-}$, 100 mM, as redox probe at 25 °C. The impedance spectra were recorded within the frequency range 0.1 Hz to 100 kHz at the formal potential of the $[Fe(CN)_6]^{3-/4-}$ redox couple. The amplitude of the alternating voltage was 5 mV. Initial potential was 0.01 V versus SCE.

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