Photocurrent response after enzymatic treatment of DNA duplexes immobilized on gold electrodes: electrochemical discrimination of 5-methylcytosine modification in DNA†

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We demonstrate a photoelectrochemical approach to the detection of the methylation status of cytosine bases in DNA. We prepared anthraquinone (AQ) photosensitizer-tethered oligodeoxynucleotide (ODN) duplexes bearing 5-methylcytosine (^{m}C) or the corresponding cytosine (C) at a restriction site of the ODN strand immobilized on gold electrodes, and measured their photocurrent responses arising from hole transport after enzymatic digestion. Treatment with *Hap*II or *Hha*I of the duplexes bearing normal C led to strand cleavage, and the photosensitizer unit was eliminated from the ODN strand immobilized on the gold electrode, exclusively reducing the photocurrent density. With a similar treatment, the duplexes bearing ^{m}C showed higher photocurrent responses arising from hole transport through the duplex. This significant difference in the photocurrent response between ^{m}C and normal C residues in DNA on the gold electrodes is potentially applicable to the detection of ^{m}C modification in DNA.

Introduction

Electrochemical devices based on the recognition and conducting properties of DNA have been developed for detecting nucleoside sequences, proteins, and small molecules.[1–5] Among the various electrochemistry-based DNA devices developed so far, the photoelectrochemical approach[3–5] has attracted a great deal of attention, because of the high sensitivity and selectivity of the detected signal as well as lower cost for easy production of the related devices.

Recently, Saito and Okamoto reported the characterization of photostimulated long range hole transport through DNA by measuring the photocurrent responses of a photosensitizer-bearing duplex immobilized on gold electrodes, and applied their photoelectrochemical system to single nucleotide polymorphisms (SNPs) typing.[3] In addition, different photoelectrochemical approaches using a CdS semiconductor nanoparticle[4a] or naphthaldiimide photosensitizer[4b] on DNA revealed the detailed mechanism of electron or hole transport through DNA film. We have also evaluated the hole transport capability of DNA duplexes and triplexes by a similar photoelectrochemical assay.[5] Based on these studies, a hole transport-induced photocurrent response arising from a DNA duplex on an electrode has become an easily measured quantity.

Methylation of cytosine is believed to cause the epigenetic repression of genetic information.[6] In this light, the establishment of a method of assessing the methylation status of specific cytosine residues in DNA is increasingly important for elucidating the biological effects of cytosine methylation. To discriminate between cytosine and 5-methylcytosine (^{m}C), various methods have been proposed by our group and others that use differences in chemical,[7–9] biochemical,[10] and photochemical reactivity.[11] Although electrochemical techniques are known to have several advantages over the protocols reported so far, there have been only a few attempts to apply such electrochemical methods to the detection of ^{m}C in DNA, *e.g.*, the measurement of the peak current arising from an exogenous redox-active indicator that binds the polymerase chain reaction (PCR) product of a methylated DNA duplex,[12a] a ferrocenylnaphthalene diimide-based electrochemical hybridization assay with methylation-specific PCR,[12b] and the direct labeling of ^{m}C in a bulged duplex with electroactive units using osmate complexation.[9b]

In this work, we present a novel photoelectrochemical approach to the discrimination between C and ^{m}C in combination with enzymatic digestion. A photosensitizer-linked DNA duplex bearing ^{m}C or C at a given restriction site of the strand was immobilized on the gold electrodes, digested with enzyme, and then subjected to measurement of its photocurrent response resulting from hole transport. We observed a high photocurrent density for the methylated duplex, whereas the control duplex bearing a normal C at the corresponding site showed a significantly reduced photocurrent density that was similar to the background level. Thus, the methylation status of DNA could be detected positively by monitoring the photocurrent response.

Results and discussion

The protocol employed for the discrimination of C and ^{m}C is outlined in Fig. 1. An oligodeoxynucleotide (ODN) possessing an anthraquinone (AQ) photosensitizer unit, which has the function of hole injection and transfer, was immobilized on gold electrodes. Subsequently, the target strand to be determined for the methylation status at the restriction site of the strand was added to

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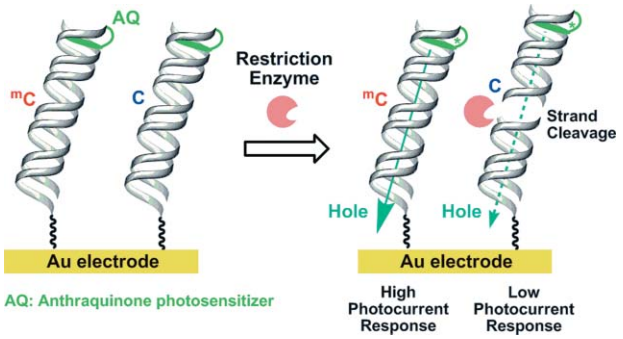

Fig. 1 The protocol for photoelectrochemical discrimination of C and ^{m}C in DNA immobilized on a gold electrode.

hybridize with the AQ-linked ODN on the electrode. The resulting duplex on the electrode was then treated with the appropriate restriction enzyme, which could digest the ODN strand at the restriction site with C but not ^{m}C. According to the protocol described above, a duplex containing C at the restriction site will show a relatively reduced photocurrent response, because the ODN strand undergoes enzymatic digestion at the C site and thereby the AQ-linked ODN fragment is eliminated from the gold electrode. In contrast, efficient photosensitized hole injection and hole transport will be preserved to cause relatively larger photocurrent response of the duplex containing ^{m}C, because the presence of ^{m}C inhibits strand cleavage by the restriction enzyme and the photosensitizing AQ linked to the ODN on the electrode is operative.

We prepared a single strand ODN with an AQ photosensitizer at the 5′-end and a thiol anchor at the 3′-end, following a previously reported method.[3b] After the spontaneous formation of a monolayer of AQ-linked thiolated ODN single strands on the surfaces of gold electrodes, the electrodes were immersed in a solution of 6-mercapto-1-hexanol to avoid any nonspecific adsorption of thiolated ODNs.[13] Duplex formation was achieved by hybridization of the AQ-linked ODNs immobilized on the gold electrodes (AQ-Probes) with the complementary ODNs (18 ± 3 pmol cm^{-2}) to be determined for the methylation status at a given target site. The photocurrents due to AQ-photoinjected hole transport through the duplex were measured after enzymatic reaction of the C- and ^{m}C-containing duplexes on the surface of the electrode, respectively. The sequences and structures of the ODNs used in this study are summarized in Fig. 2.

We initially investigated a 17-mer duplex containing C (AQ-Probe 1/ODN 1 (C)) or ^{m}C (AQ-Probe 1/ODN 1 (^{m}C)) at the *Hap*II recognition site (5′-CXGG-3′, where X = C or ^{m}C) in a partial sequence of the human p53 gene corresponding to codons 246–250 of exon 8. According to the protocol described above, a DNA-labeled gold electrode was prepared, followed by enzymatic treatment at 23 °C for 1 h with 1 unit of *Hap*II in 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 1 mM dithiothreitol. After the enzymatic reaction, the photocurrent measurements were carried out in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl at 20 °C, using 365 ± 5 nm UV light at a power density of 13.0 ± 0.3 mV cm^{-2} and an applied potential of 500 mV *vs.* SCE. Fig. 3a and 3b show representative cathodic photocurrent responses, which appeared immediately upon irradiation of the common AQ-photosensitizer

AQ-Probe 1: 5′-AU^{AQ}G AAC CGG AGG CCC AT-(CH_2)-SAu 3′
AQ-Probe 2: 5′-CU^{AQ}C TGT GCG CCG GTC TCT-(CH_2)-SAu 3′
ODN 1(X): 5′-ATG GGC CTC XGG TTC AT-3′
ODN 2(X): 5′-AGA GAC XGG CGC ACA GAG-3′
ODN 3(^{m}C): 5′-AGA GAC CGG ^{m}CGC ACA GAG-3′
ODN 4(X): 5′-(15mer)-AGA GAC XGG CGC ACA GAG-(54mer)-3′
F-ODN(X): 5′-F-AGA GAC XGG CGC ACA GAG-D-3′
X = C or ^{m}C

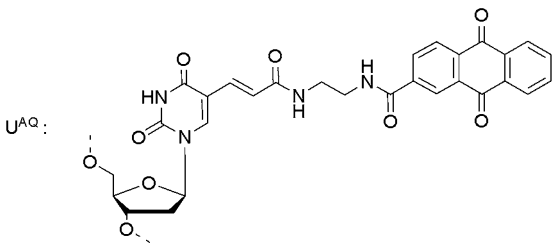

Fig. 2 Sequences and structures of ODNs used in this study. The "F" and "D" of F-ODNs denote fluorescein and dabcyl groups, respectively.

linked to both duplexes. It is striking that the photocurrent response of the C-containing duplex (AQ-Probe 1/ODN 1(C)) was much smaller than that of the ^{m}C-containing duplex (AQ-Probe 1/ODN 1(^{m}C)). The photocurrent densities (current per electrode area) observed for the digested C-containing duplex and the undigested ^{m}C-containing duplex were 178 ± 13 and 271 ± 15 nA cm^{-2}, respectively. The photocurrent density (175 ± 18 nA cm^{-2}) observed upon similar UV irradiation of an AQ-linked thiolated ODN single strand indicates that the enzymatic treatment of the unmethylated duplex could suppress an almost quantitative amount of the photocurrent response.[14] Thus, the methylation status of cytosine was detectable by monitoring the photocurrent response in conjunction with restriction enzyme treatment.

Compared to control photocurrent measurements for duplexes containing ^{m}C and C, respectively without enzymatic treatment, the photocurrent density of the AQ-Probe 1/ODN 1(^{m}C) duplex after the enzymatic reaction with *Hap*II seemed to be slightly small (Fig. 3c). Recently, Barton and co-workers have shown that the binding of the restriction endonuclease *Pvu*II to its methylated target causes a small decrease in current flow in an electrochemical assay using daunomycine-tethered DNA.[15] In accord with this result, our observation of a slight decrease in the photocurrent of the AQ-Probe 1/ODN 1(^{m}C) duplex may be attributed to the occurrence of *Hap*II binding to the methylated target.

The photocurrent response of DNA with a different DNA sequence and the *Hap*II recognition site was also measured. We prepared AQ-tethered duplexes (AQ-Probe 2/ODN 2(^{m}C) and AQ-Probe 2/ODN 2(C)) on gold electrodes, which corresponded to codons 280–285 of exon 8 in the human p53 gene. After treatment with *Hap*II, photocurrent measurements were conducted in a similar manner as described above. As shown in Fig. 4, treatment of the AQ-Probe 2/ODN 2(C) duplex with *Hap*II led to a significant suppression of the photocurrent response (203 ± 17 nA cm^{-2}), while the response for the AQ-Probe 2/ODN 2(^{m}C) duplex was preserved (281 ± 17 nA cm^{-2}), as was observed for the AQ-Probe 1/ODN 1(^{m}C) duplex.[16] To apply a different restriction enzyme to the present system, we

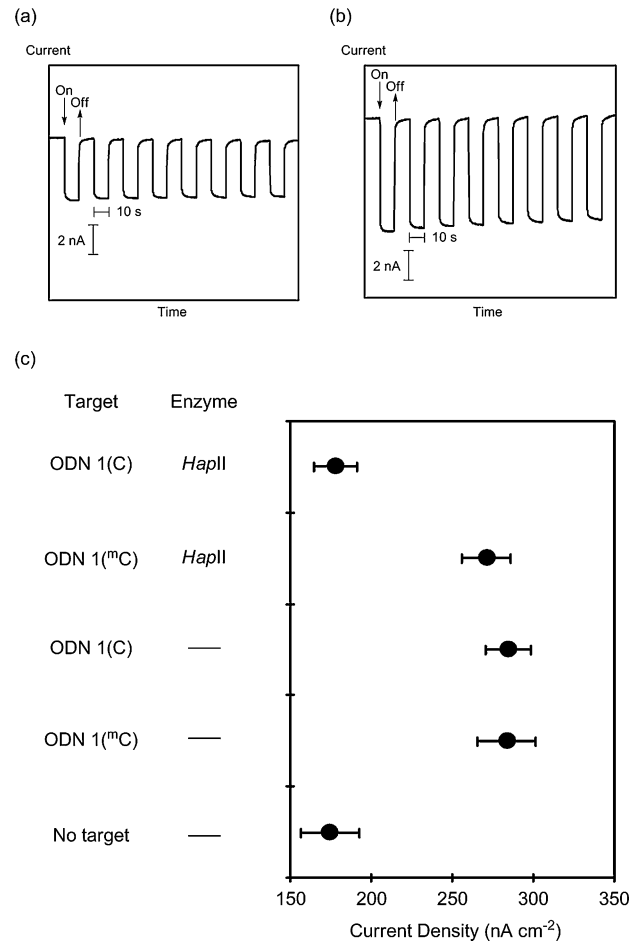

Fig. 3 (a) Photocurrent response of AQ-Probe 1/ODN 1(C) duplex immobilized on a gold electrode after treatment of the duplex with 1 unit of *Hap*II at 23 °C for 1 h on the electrode. The photocurrent measurements were carried out at an applied potential of 500 mV *vs.* SCE upon photoirradiation with 365 ± 5 nm light (13.0 mW cm^{-2}) at 20 °C in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl. (b) Photocurrent response of AQ-Probe 1/ODN 1(^{m}C) duplex under the same conditions. (c) Photocurrent densities of AQ-Probe 1/ODN 1(X) duplexes before and after enzymatic treatment with *Hap*II. Each error bar represents the SE calculated from ten experimental results that were corrected using different freshly prepared gold electrodes.

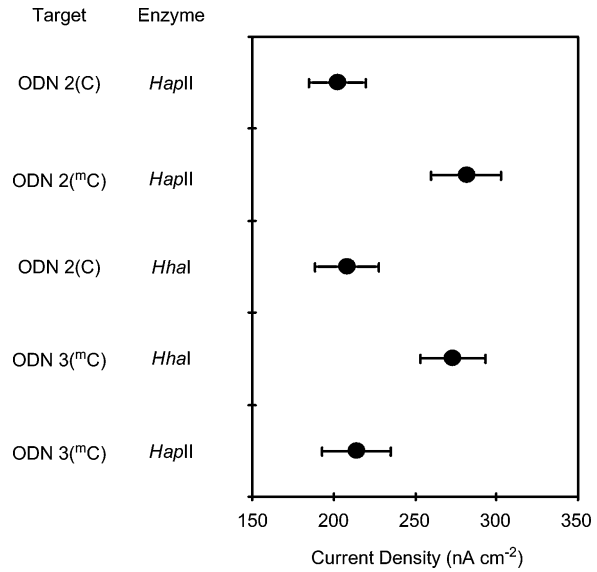

Fig. 4 Photocurrent densities of duplexes consisting of AQ-Probe 2 and its target ODN (ODN 2(X) or ODN 3(^{m}C)), as evaluated at an applied potential of 500 mV *vs.* SCE upon photoirradiation with 365 ± 5 nm light (13.0 mW cm^{-2}) at 20 °C in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl, after treatment of the duplexes immobilized on the electrode with 1 unit of *Hap*II or *Hha*I at 23 °C for 1 h. Each error bar represents the SE calculated from ten experimental results that were corrected using different freshly prepared gold electrodes.

also prepared a duplex of AQ-Probe 2/ODN 3(^{m}C) on the electrode, in which the ^{m}C was arranged at the recognition site of *Hha*I (5′-GCGC-3′). After treatment with *Hha*I, we observed clear differences in the photocurrent response between AQ-Probe 2/modified ODN 3(^{m}C) and AQ-Probe 2/normal ODN 2(C). In a control experiment, enzymatic treatment of the AQ-Probe 2/ODN 3(^{m}C) duplex with the noncompliant enzyme *Hap*II led to the suppression of the photocurrent due to strand cleavage at the *Hap*II recognition site, indicating that employment of the best-suited enzyme for the methylated target site allows us to site-specifically discriminate modified ^{m}C from normal C in a given DNA.

To confirm the occurrence of enzymatic strand cleavage at the normal C target site causing exclusive suppression of the photocurrent response for duplexes without methylation, we performed control experiments with F-ODNs (C and ^{m}C) possessing a fluorophore (fluorescein) and a quencher (dabcyl group) at the strand end, in which the fluorescence emissions were monitored after enzymatic digestion of the F-ODNs (C and ^{m}C)/AQ-Probe 2 duplexes. As shown in Fig. 5a, significant enhancement of the fluorescence intensity was observed for the F-ODN (C)/AQ-Probe 2 duplex after the *Hap*II treatment, whereas the fluorescence of the F-ODN (^{m}C)/AQ-Probe 2 duplex after a similar treatment was weak. These results strongly suggest that enzymatic strand cleavage occurred exclusively at the unmethylated recognition site, leading to the separation of the fluorophore from its neighbouring quencher, with intense fluorescence emission. We also characterized this enzymatic reaction in further detail, using gel electrophoretic analysis of the restriction products. As shown in Fig. 5b, the major cleavage band assigned to the expected restriction products was observed after digestion of the F-ODN(C)/AQ-Probe 2 duplex with the *Hap*II, while a negligible amount of strand cleavage occurred when the F-ODN(^{m}C)/AQ-Probe 2 duplex was treated similarly. These features in the electrophoretic analysis are entirely in accord with the above fluorometric study, thus leading to the conclusion that enzymatic strand cleavage at the unmethylated target site is responsible for the suppression of the photocurrent response.[17]

For establishing the utility of the present system, a further attempt was made to evaluate the photoelectrochemical discrimination between normal C and modified ^{m}C in a long sequence (87 mer) of the human p53 gene corresponding to codons 275–303 of exon 8, in which the methylation status at codon 282 was examined.[18] According to the protocol described above, a partial DNA duplex consisting of ODN 4(X)/AQ-Probe 2 was prepared on gold electrodes, and the photocurrent was measured after *Hap*II treatment. As shown in Fig. S2,† a high photocurrent

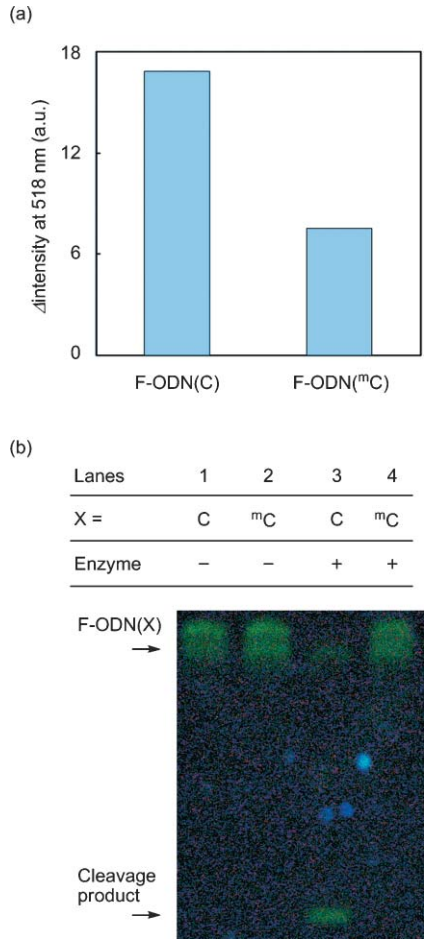

Fig. 5 (a) Changes in the fluorescent intensity of F-ODN(C or ^{m}C)/AQ-Probe 2 duplexes after treatment with *Hap* II. The fluorescence emission was measured at 518 nm upon excitation at 495 nm. *Δ*Intensity is a differential value derived from subtraction of background without *Hap*II treatment from the fluorescence intensity of the *Hap*II treated sample. (b) A denaturing polyacrylamide gel picture after electrophoresis for F-ODNs. F-ODN(C or ^{m}C)/AQ-Probe 2 duplexes were incubated for 1 h in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of *Hap*II, respectively: lanes 1 and 3, F-ODN(C)/AQ-Probe 2 duplex; lanes 2 and 4, F-ODN(^{m}C)/AQ-Probe 2 duplex.

response was preserved for the duplex methylated at codon 282 (299 ± 29 nA cm^{-2}), whereas the photocurrent density of the corresponding duplex with a normal C at codon 282 was significantly reduced to the background level (195 ± 23 nA cm^{-2}). Thus, ^{m}C can be discriminated clearly from normal cytosine in DNA by monitoring the photocurrent due to photochemically-injected hole transport through DNA on gold electrodes, in combination with enzymatic digestion.

In this study, we could successfully develop a photoelectrochemical system to discriminate between ^{m}C and normal C in a target DNA strand. However, the present protocol has some points to be considered upon its application. Firstly, the present assay requires single-stranded target DNA, which will be able to hybridize with the synthetic ODN immobilized on gold electrodes, although the real gene is present in duplex form. One of the key strategies for the methylation analysis of a gene is employment of peptide nucleic acids (PNA).[10b,19] In this context, PNAs have very high affinity to complementary nucleic acids because of the lack of repulsive phosphate–phosphate interactions. Hybridization of a PNA strand to double-stranded DNA results in the formation of a strand displacement complex. Such a displaced strand allows binding with another complementary strand to form a DNA–PNA complex referred to as a PD-loop. Thus, the single-stranded region in genomic DNA created by PNA can apply to the present protocol. Secondly, the system can apply to identification of the methylation status in DNA, but not to discrimination between sequences that differ by single base changes: *e.g.* the target DNA bearing a T, A or G base instead of C would lead to low photocurrent responses after enzymatic digestion similar to the corresponding responses of a digested C-containing target. Although the restriction enzyme, which is compliant with the C-containing duplex, cannot cleave the DNA bearing T, A or G, the disruption of the π-stacking array by mismatched base pairs may strongly influence the photocurrent intensity.[3b,4b] Thus, the present system can only apply to the positive detection of ^{m}C modification in DNA.

Conclusions

In summary, we have demonstrated a photoelectrochemical approach to the discrimination between C and ^{m}C in DNA, using the photosensitizer-injected hole transfer properties of DNA immobilized on gold electrodes in combination with enzymatic digestion. Upon digestion of the duplex immobilized on the electrodes, a significant suppression of the photocurrent response was observed for duplexes with a normal C at the restriction site. This reduced photocurrent was attributable to strand cleavage at the C target site, by which the AQ photosensitizer on the duplex was eliminated from the electrode. In contrast, the duplex containing ^{m}C at the target site did not undergo such an enzymatic digestion and therefore the photocurrent response was preserved.

Although the present system has a strong potential for the photoelectrochemical identification of methylation status, establishment of a highly sensitive detection system is further required for practical utilization to identify the methylation status in DNA at attomol level. Our current study is focused on establishment of a protocol with high sensitivity and generality by optimizing the length of the photosensitizer-linked probe sequence and the size of the electrode to fabricate DNA chips.

Experimental

General methods

The reagents for the DNA synthesizer were purchased from Glen Research. *Hap*II and *Hha*I restriction enzymes were purchased from TAKARA BIO. All aqueous solutions were prepared using purified water (YAMATO, WR600A). Mass spectrometry of oligonucleotides (ODNs) was performed with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Perseptive Voyager Elite, acceleration voltage 21 kV, negative mode) with 2′,3′,4′-trihydroxyacetophenone as a matrix, using T_8 ([M − H]$^-$ 2370.61), T_{17} ([M − H]$^-$ 5108.37) and T_{27} ([M − H]$^-$ 8150.33) as the internal standards. Reversed phase HPLC was performed with a Shimadzu 6A, HITACHI D-7000 HPLC system or HITACHI L-2400. Sample solutions

were injected on a reversed phase column (Inertsil ODS-3, GL Sciences Inc., ϕ 4.6 mm × 250 mm or ϕ 10 mm × 250 mm). The column eluents were monitored by UV absorbance at 260 nm. Anthraquinone (AQ)-linked thiolated ODNs were synthesized as described previously.[3,5] Modified ODNs possessing fluorescein and dabcyl groups (F-ODNs) were synthesized using standard phosphoroamidite chemistry.

DNA characterization

Each ODN synthesized in this study was characterized by MALDI-TOF Mass: AQ-Probe 1; calcd. for 5731.91, found 5732.84, AQ-Probe 2; calcd. for 5961.01, found 5961.83, F-ODN(C); calcd. for 6566.5, found 6566.7, F-ODN(^{m}C); calcd. for 6580.5, found 6580.0.

Immobilization of AQ-linked thiolated ODN on a gold electrode

A gold electrode with an area of 2 mm^2 (BAS) was used in this study. Prior to ODN immobilization, the gold electrode was polished with BAS polishing diamond suspension and alumina suspension, and then washed with deionized water. The polished electrode was soaked in boiling 2 M potassium hydroxide for 3 h and washed with deionized water. Following this treatment, the electrode was further soaked in concentrated nitric acid for 1 h and washed with deionized water. For chemisorption of ODN, a 1 μL solution of 10 μM AQ-linked thiolated ODN was placed on a gold electrode turned upside-down and the opening of the electrode vessel was then stuffed with a rubber stopper to avoid evaporation of the solvent. After leaving the assembly for 2 h at room temperature, a 1 μL solution of 10 mM 6-mercaptohexanol in 10 mM Tris-EDTA buffer (pH 8.0) was placed on a gold electrode turned upside-down for masking of the gold surface and the opening of electrode vessel was then stuffed with a rubber stopper to avoid evaporation of the solvent. After leaving the assembly for 1 h at room temperature, the electrode was carefully washed with a small amount of deionized water. For the duplex formation with target ODNs immobilized on a gold electrode, a 1 μL aqueous solution of 10 μM of the target-ODN was placed on a gold electrode turned upside-down and the opening electrode vessel was then stuffed with a rubber stopper to avoid evaporation of the solvent. The assembly was left for 2 h at 4 °C prior to photocurrent measurement.

General procedure for the reaction of an AQ-linked ODN duplex immobilized on gold electrodes with a restriction enzyme

For the enzymatic reaction at target base sequences of the AQ-linked ODN duplex immobilized on a gold electrode, a 1 μL solution of *Hap*II restriction enzyme (1 unit) in 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$ and 10 mM dithiothreitol or a 1 μL solution of *Hha*I restriction enzyme (1 unit) in 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM NaCl was placed on a gold electrode turned upside-down and the opening of the electrode vessel was then stuffed with a rubber stopper to avoid evaporation of the solvent, and the electrode was kept standing for 1 h at 23 °C. After washing with deionized water, photocurrent measurements were performed.

Photocurrent measurements

Photocurrent measurements were performed in a one-compartment Pyrex cell at an applied potential of 500 mV *vs.* SCE under illumination by a 200 W UV lamp (Sumida YLT-MX200) with monochromatic exciting light through a 365 ± 5 nm band pass filter (ϕ 25 mm, Asahi Bunko). The cell for photocurrent measurement consisted of a three-electrode arrangement (ALS, model 660B) of a modified Au working electrode (electrode area, 2 mm^2), a platinum counter electrode and an SCE reference electrode at 20 °C. The light intensity was monitored by an optical powermeter (Ushio UIT-150). The photocurrent was measured in 2 mM sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl with $\lambda = 365 \pm 5$ nm light with a power density of 13.0 mW cm^{-2}.

Determination of surface density of ODNs on a gold electrode

The amount of ODN immobilized on the electrode was determined using a chronocoulometric assay in the presence of ruthenium(III) hexaammine.[20]

Fluorescence measurement of the enzymatic digestion reaction of F-ODN duplexes with *Hap*II

Solutions (100 μL) of 1 μM AQ-linked thiolated ODN and 1 μM F-ODNs in 10 mM Tris-HCl pH 7.5 containing 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) with *Hap*II (20 units) were incubated at 23 °C for 30 min. The reaction mixtures were quenched by heating for 3 min at 90 °C and were quickly chilled on ice, and then diluted with 250 μL of deionized water. After dilution, fluorescence intensity (518 nm) was measured upon excitation at 495 nm.

PAGE analysis of the enzymatic digestion reaction of F-ODN duplexes with *Hap*II

Solutions (100 μL) of 2 μM AQ-linked thiolated ODN and 2 μM F-ODNs in 10 mM Tris-HCl pH 7.5 containing 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) with *Hap*II (20 units) were incubated at 23 °C for 1 h. After the incubation, all reaction mixtures were precipitated with addition of 20 μL of 3 M sodium acetate and 800 μL of ethanol. The precipitated DNA was washed with 100 μL of 80% cold ethanol and then dried *in vacuo*. The dried DNA pellets were resuspended in 5 μL of a loading buffer (a solution of 8 M urea and 40% sucrose). The samples were loaded onto 20% of polyacrylamide/7 M urea sequencing gels and electrophoresed at 60 W for 60 min. Fluorescent bands were visualized over a UV transilluminator (365 nm) and images were recorded using a CANON Power Shot G6 digital camera.

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