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Cleavage at 5-methylcytosine in DNA by photosensitized oxidation with 2-methyl-1,4-naphthoquinone tethered oligodeoxynucleotides

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Abstract—Photosensitized one-electron oxidation of 5-methylcytosine in DNA by 2-methyl-1,4-naphthoquinone, attached to 5′-end of an oligodeoxynucleotide strand, produced 5-formylcytosine and led to selective DNA strand cleavage at the original 5-methylcytosine configuration. This specified photoreaction is useful for positive display of 5-methylcytosine in DNA on a sequencing gel.

Cytosine methylation at 5-position in genomic DNA is believed to alter DNA–protein interactions assisting in gene regulation and silencing, thereby causing various human diseases including cancer.[1–8] In this view, identification of the methylation status of cytosine residues in DNA becomes increasingly significant to get molecular insights into the biochemical function of cytosine methylation. Recently, several protocols including Maxam–Gilbert chemical modification, sodium bisulfite modification, or enzymatic digestion of peptide nucleic acids–DNA complex, have been proposed to detect 5-methylcytosine (5-^{m}C).[9–13] These protocols were based on the differences in reactivity between 5-^{m}C and normal cytosine toward chemical modification and enzymatic digestion. Thus, understanding of the reaction characteristics of 5-^{m}C in DNA is important for the development of a convenient and general protocol for detecting 5-^{m}C.

2-Methyl-1,4-naphthoquinone (**NQ**), a component of vitamin K_3, is known to photosensitize DNA base damages by a one-electron oxidation mechanism.[14,15] In the **NQ** photosensitized oxidation of 5-methyl-2′-deoxycytidine, the major products were identified to be 5-formyl-2′-deoxycytidine and 5-(hydroxymethyl)-2′-deoxycytidine.[16] The characteristics of this reaction suggest that 5-^{m}C may show different reactivity from normal cytosine in DNA towards **NQ** photosensitized oxidation.

We report herein an efficient cleavage at 5-^{m}C in oligodeoxynucleotides (**ODN**s) using photosensitization with **NQ**-tethered **ODN** (**NQ-ODN 1**) as synthesized to target a specified base sequence in DNA. Upon photoirradiation at λ_{ex} = 312 nm a stable duplex of **NQ-ODN 1** with complementary **ODN** bearing a 5-^{m}C configuration (**ODN 3**) underwent oxidation of the 5-^{m}C residue to produce 5-formylcytosine and of the guanine residue. Treatment with hot piperidine after photoirradiation led to strand cleavage at the resulting 5-formylcytosine residue, thus a positive band was observed at the position of original 5-^{m}C on a sequencing gel. In contrast to the behavior of 5-^{m}C, the corresponding cytosine residue did not result in such a strand cleavage. This specified photoreaction is potentially applicable for positive display of 5-^{m}C configuration generated in DNA on a sequencing gel.

The synthesis of **NQ-ODN 1** is outlined in Scheme 1. Reaction of 2-methyl-1,4-naphthoquinone **NQ** with succinic acid gave carboxylated derivative of **NQ** via oxidative decarboxylation of succinic acid.[17] Carboxylic acid **1** was coupled with *N*-hydroxysuccinimide to give *N*-hydroxysuccinimidyl ester **2**.[18] Coupling of **2** with modified **ODN 1** possessing an aminohexyl linker at the 5′-end resulted in **NQ-ODN 1**.[19] The crude **NQ-ODN 1** was purified by reversed phase HPLC and the incorporation of **NQ** chromophore into **ODN 1** was confirmed

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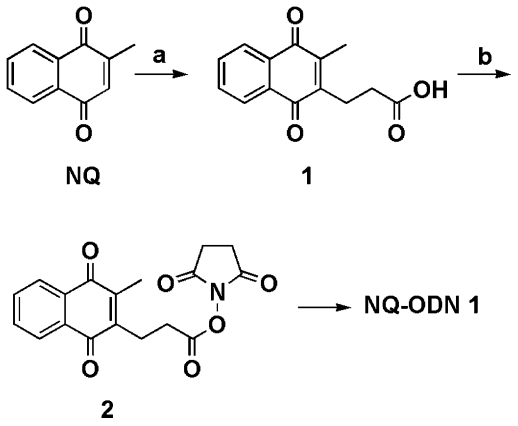
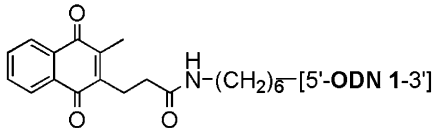


Scheme 1. Reagents and conditions: (a) succinic acid, $AgNO_3$ catalytic, $(NH_4)_2S_2O_8$, aq 30% acetonitrile, 65 °C, 5 h (63%); (b) DCC, NHS, DMF, rt, 24 h (60%); (c) $H_2N(CH_2)_6$-[5′-**ODN 1**-3′], satd $NaHCO_3$ aq, acetonitrile–water (1:1), rt, 20 h.

by enzymatic digestion and MALDI-TOF mass spectroscopy (calcd 7624.05, found 7624.11).

ODNs used in this study are listed in Figure 1. The stability of four couples of **ODN** duplexes were evaluated by measuring the melting temperatures (T_m) as shown in Figure 1. The duplexes consisting of **NQ-ODN 1** showed slightly higher T_m values than the corresponding duplexes without **NQ** chromophore. The stabilization of duplex by **NQ** chromophore tethered at the strand end is probably due to the π-stacking of a hydrophobic planar ring of **NQ** with the flanking bases.

Photosensitized oxidation (λ_{ex} = 312 nm) of **NQ**-C-duplex or **NQ**-^{m}C-duplex consisting of a common **NQ-ODN 1** with ^{32}P-5′-end labeled **ODN 2** or **ODN 3**, respectively, was performed in sodium cacodylate buffer (pH 7.0) containing 20 mM NaCl at 0 °C. The reaction was analyzed by polyacrylamide gel electrophoresis after



ODN 1: 5′-d(TTTAGCCATTCCTTCTACCTTGAT)-3′

3′-d(AAAAXAAATCGGTAAGGAAGATGGAACTA)-5′

ODN 2: X = C; **ODN 3**: X = ^{m}C

		T_m (°C)
NQ-C-duplex:	**NQ-ODN 1 / ODN2**	52.7
NQ-^{m}C-duplex:	**NQ-ODN 1 / ODN 3**	51.4
C-duplex:	**ODN 1 / ODN2**	49.1
^{m}C-duplex:	**ODN 1 / ODN 3**	49.0

Figure 1. Sequences of the oligodeoxynucleotides and T_m of the duplexes with **NQ** chromophore.

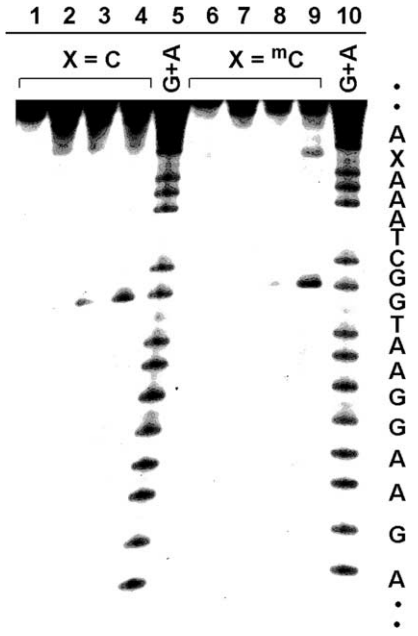


Figure 2. A representative autoradiogram of denaturing gel electrophoresis for ^{32}P-5′-end labeled **ODN 2** and **ODN 3** after 312 nm photoirradiation in the presence of 20 mM NaCl in 2 mM sodium cacodylate buffer (pH 7.0) at 0 °C for 0 h (lanes 2 and 7), 1 h (lanes 3 and 8), and 2 h (lanes 1, 4, 6, and 9). After hot piperidine treatment (90 °C, 20 min), the samples were electrophoresed through a denaturing 15% polyacrylamide/7 M urea: lane 1, C-duplex; lanes 2–4, **NQ**-C-duplex; lane 6, ^{m}C-duplex; lanes 7–9, **NQ**-^{m}C-duplex; lanes 5 and 10, Maxam–Gilbert G + A sequencing lanes.

treatment with hot piperidine.[20] A representative gel picture was shown in Figure 2. Photoirradiation of **NQ** chromophore in both duplexes for 2 h led to efficient strand cleavage at guanine doublets that were demonstrated to be an efficient electron donating site in DNA.[21] A striking result was the intense cleavage at 5-$^{m}C_{25}$ in the **NQ**-^{m}C-duplex, while we could observe substantially no cleavage at C_{25} in the control **NQ**-C-duplex. In the separate experiments, we also determined the photoreactivity of ^{m}C-duplex in the presence of DNA-free monomeric sensitizer **NQ** at the same chromophore concentration as in **NQ-ODN 1**, and that of equimolar mixture of **NQ-ODN 1** and noncomplementary **ODN 4** bearing 5-^{m}C. In both photosensitized reactions for 2 h the strand cleavages at 5-^{m}C were suppressed to the background levels. It is therefore likely that arrangement of the 5-^{m}C residue in the vicinity of **NQ** chromophore as occurring in the **NQ**-^{m}C-duplex would be more favorable than its separation far from the chromophore for the photosensitized oxidation of 5-^{m}C residue.

In order to characterize the oxidation products derived from 5-^{m}C in DNA, **NQ**-^{m}C-duplex was similarly irradiated at λ_{ex} = 312 nm for 2 h in sodium cacodylate buffer (pH 7.0) at 0 °C and then subjected to digestion by enzyme. ESI/MS analysis of the resulting reaction mixture after purification by HPLC indicated the formation of 5-formyl-2′-deoxycytidine ($[M]^-$, 255) as one of the oxidants of 5-methyl-2′-deoxycytidine. Based on this result,

it is reasonable to presume that 5-^{m}C residue in **ODN 3** may be converted to alkali-labile 5-formylcytosine by photosensitized oxidation with **NQ** chromophore in the **NQ-ODN 1**, in a similar manner as in the **NQ** photosensitized oxidation of monomeric 5-^{m}C reported previously.[17,22] Since 5-^{m}C residue in the **ODN 3** was arranged to have contact with **NQ** in the duplex formation with **NQ-ODN 1**, the primary photochemical process is most likely to involve direct one-electron transfer from 5-^{m}C to the triplet excited state of **NQ** (^{3}**NQ***).[23] Thus, although guanine doublets are simultaneously oxidized, occurrence of 5-^{m}C in DNA can be identified distinguishably from a cytosine residue by monitoring the photosensitized oxidative cleavage of 5-^{m}C-induced DNA, using appropriate **NQ**-tethered oligodeoxynucleotides.

In summary, we presented a great difference in reactivity between 5-^{m}C and normal cytosine residues in DNA, toward photosensitized oxidation with **NQ**-tethered oligodeoxynucleotides. An oligodeoxynucleotide bearing **NQ** chromophore (**NQ-ODN 1**) was synthesized and demonstrated to form quite stable duplexes with its complementary oligodeoxynucleotide strands. The photosensitization experiment of **NQ**-tethered duplex revealed that 5-^{m}C was efficiently oxidized to 5-formylcytosine, accompanied by the oxidation of guanine doublets. Upon treatment with hot piperidine, the photosensitized oxidation site at 5-^{m}C could be displayed as a positive band on the sequencing gel.

Our current study is focused on monitoring of the photosensitized oxidation of 5-^{m}C in CpG rich areas where cytosine methylation in mammals is apt to occur, using a family of **NQ**-tethered **ODN**s with varying base sequences.

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18. Compound **2**: Mp 48–50 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03–7.99 (m, 2H), 7.66–7.61 (m, 2H), 3.00 (t, J = 7.5 Hz, 2H), 2.82 (t, J = 7.8 Hz, 2H), 2.75 (s, 4H), 2.16 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.6, 168.7, 167.4, 145.1, 143.2, 133.4, 131.8, 126.2, 29.6, 25.6, 22.6, 13.0; FABMS (NBA) *m/z* 288 [$(M+H)^+$]; HRMS calcd for $C_{16}H_{18}NO_4$ [$(M+H)^+$] 288.1236, found 288.1235.
19. Preparation of **NQ-ODN 1**: To a solution (total volume 50 μL) of **ODN 1** possessing an aminohexyl linker at the 5′-end was added the solution of compound **2** (68 μg, 0.2 μmol) and satd $NaHCO_3$ aq (20 μL), and incubated at 25 °C overnight. The reaction mixture were purified by reversed phase HPLC (elution with a solvent mixture of 0.1 M TEAA, pH 7.0, linear gradient over 60 min from 0% to 30% acetonitrile and over 80 min from 30% to 100% acetonitrile at a flow rate 3.0 mL/min) to give **NQ-ODN 1**.
20. General procedures for photoreaction and PAGE analysis: ^{32}P-5′-end-labeled **ODN**s (<400 nM strand concentration) and cold **ODN**s (2 μM) were hybridized by their complimentary strands (2 μM) in 2 mM sodium cacodylate containing 20 mM NaCl buffer (pH 7.0). The ^{32}P-5′-end-labeled duplex was irradiated at 312 nm with a transilluminator at 0 °C. After irradiation, all reaction mixtures were precipitated with addition of 10 μL of herring sperm DNA (1 mg/mL), 10 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 precipitated DNA was resolved in 50 μL of 10% piperidine (v/v), heated at 90 °C for 20 min and concentrated. The radioactivity of the samples was assayed using an Aloka 1000 liquid scintillation counter and the dried DNA pellets were resuspended in 80% formamide loading buffer (a solution of 80% formamide (v/v), 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). All reactions were heat-denatured at 90 °C for 3 min and quickly chilled on ice. The samples ($3–5 \times 10^3$ cpm) were load onto 15% of polyacrylamide/7 M urea sequencing gels and electrophoresed at 1900 V for 60–90 min transferred to a cassette and stored at −80 °C with Fuji X-ray film (RX-U). The gels were analyzed by autoradiography with ATTO densitograph software library (version 3.0). The intensity of the spots resulting from piperidine treatment was determined by volume integration.
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23. Adenine has been reported to undergo **NQ**-photosensitized oxidation with one order of magnitude less efficiency than guanine (see Ref. 14), suggesting a possibility of alternative adenine-hopping mechanism (Kawai, K.; Takada, T.; Tojo, S.; Majima, T. *J. Am. Chem. Soc.*

2003, *125*, 16198), by which one-electron oxidation of adjacent adenine moiety by ^{3}**NQ*** occurs and the resulting hole migrates to oxidize 5-^{m}C and guanine residues. In a separate photoreaction of the 24-mer duplex of **NQ-ODN 1** with **ODN 5** (5′-ATCAAGGTAGAAGGAATGG^{m}C-TAAA-3′), we observed efficient cleavages at guanine doublets, but not at 5-$^{m}C_{20}$ due to the absence of direct contact with **NQ**.