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The pH Effect on the Naphthoquinone-Photosensitized Oxidation of 5-Methylcytosine

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Abstract: The pH effect on the one-electron photooxidation of 5-methyl-2′-deoxycytidine (d^mC) by sensitization with 2-methyl-1,4-naphthoquinone (NQ) was investigated. Photoirradiation of an aqueous solution containing d^mC and NQ under slightly acidic conditions of pH 5.0 efficiently produced 5-formyl-2′-deoxycytidine, whereas similar NQ-photosensitized oxidation of d^mC proceeded to a lesser extent under more acidic or basic conditions. Fluorescence-quenching experiments revealed that the less-efficient photooxidation at pH values below 4.5 is attributed to the decreased rate of one-electron oxidation of d^mC owing to protonation at the N(3)-position. The NQ-photosensitized oxidation of an N(4)-dimethyl-substituted d^mC derivative under various pH conditions also suggests that the pH change in the range of 5.0 to 8.0 may be responsible for a reversible deprotonation–protonation equilibrium at the N(4)-exocyclic amino group of the d^mC radical cation. In accord with the photochemical reactivity of monomeric d^mC, the 5-methylcytosine residue in oligodeoxynucleotides was oxidized efficiently by photoexcited NQ-tethered oligodeoxynucleotides under slightly acidic conditions to form an alkali-labile 5-formylcytosine residue.

Keywords: methylcytosine · DNA methylation · epigenetic analysis · nucleic acids · photooxidation

Introduction

Cytosine methylation is a physiological modification that takes place after DNA replication. In the course of such a modification, a methyl group is transferred from (*S*)-adenosylmethionine to the C5 position of cytosine by DNA methyltransferases.[1,2] Cytosine methylation is believed to play a number of important biological roles, typically the epigenetic repression of genetic information, although it is not fully elucidated.[1,2] For a better understanding of the biological effects of cytosine methylation, there is increasing interest in developing an effective method to assess the methylation status of specific cytosine residues in genomes. Various methods for identification and analysis of cytosine methylation have been reported in which differences in chemical,[3–7] biological,[8] or photochemical[9] reactivity between cytosine (C) and 5-methylcytosine (^mC) are evaluated.

At present, the bisulfite method[4–5] is most commonly used among various different protocols for the detection of cytosine methylation. This protocol is based on the sodium bisulfite modification of normal C but not ^mC and allows the clear discrimination between C and ^mC in genomic DNA with high selectivity. There are, however, practical drawbacks to the method including complicated procedures and long reaction periods for chemical modification. To replace the bisulfite method, several chemical protocols based on the direct modification of ^mC but not C have been reported. Okamoto et al. showed that treatment of a single ^mC bulge of duplex DNA with osmium tetroxide produces an ^mC–osmium adduct in a sequence-selective manner.[6] Bareyt and Carell reported that the ^mC in duplex DNA is oxidized selectively by pentavalent vanadium species or sodium periodate in the presence of lithium bromide.[7] These reactions have been applied to sequence-specific DNA-methylation analysis.

Recently, we proposed a protocol for discriminating ^mC based on the one-electron photooxidation of ^mC in a given oligodeoxynucleotide (ODN) sensitized by 2-methyl-1,4-naphthoquinone (NQ)-tethered ODN.[9] Photoirradiation of an NQ-tethered duplex in an aqueous solution induced the

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efficient one-electron oxidation of the ^{m}C residue to form 5-formylcytosine when the NQ chromophore was arranged so as to be in close contact with the target ^{m}C.[9a,b] Subsequent treatment of the photoirradiated aqueous solution with hot piperidine led to exclusive strand cleavage at the original ^{m}C site. In contrast, an ODN analogue, which replaced ^{m}C with normal C, thymine (T), adenine (A), or guanine (G) underwent less oxidative strand cleavage at the alternative target site probably because of charge-transfer and charge-recombination processes between the base radical cation and the NQ radical anion. Suppressed strand cleavages at C and T were also explained by less-efficient NQ-sensitized photooxidation to form the corresponding radical cations owing to the considerably smaller free-energy change of charge separation for photooxidation of C and T by excited NQ.[9b] In addition, well-designed incorporation of the NQ chromophore into the interior of ODN suppressed the competitive strand cleavage exclusively at consecutive G bases, which occurred as a result of positive charge transfer. Thus, such a striking photooxidative reactivity leading to strand cleavage allowed us to detect the target ^{m}C as a positive band on the sequencing gel.

Although the NQ-photosensitized oxidation accompanied by selective strand cleavage at ^{m}C in DNA is an attractive method for identification of the methylation site, relatively lower sensitivity of the detection method owing to reduced efficiency in the photooxidation of ^{m}C still remains as a major issue that must be improved to establish a status of general availability. Herein, we characterized the pH effect on the NQ-photosensitized one-electron oxidation of 5-methyl-2′-deoxycytidine (d^{m}C) to obtain insight into the design of photochemical systems involving the ^{m}C-selective strand cleavage with high sensitivity. A pH change is among the potential external triggers for regulation of various photoreactions. Upon photoirradiation of monomeric d^{m}C in the presence of NQ under various pH conditions, a significant pH-dependent formation of 5-formyl-2′-deoxycytidine (d^{f}C) was observed. We confirmed that the formation of d^{f}C occurs with maximum efficiency at pH 5.0, whereas the efficiency of photooxidation was lower under more acidic or basic conditions. In addition, we investigated the NQ-photosensitized oxidative cleavage reaction of ^{m}C in a duplex in which the NQ chromophore was incorporated into the strand for immobilization at a specific position, thus obtaining evidence that optimization of the pH environment in the photosensitized oxidation results in much larger amount of strand cleavage at ^{m}C in DNA.

Results and Discussion

We initially performed the photooxidation of d^{m}C by sensitization with NQ at various pH values. Aerobic solutions of d^{m}C (200 μM) and NQ (200 μM) in 2 mM sodium cacodylate buffer solution containing 20 mM NaCl and 10% acetonitrile at a pH range of 4.0 to 8.0 were photoirradiated by using 312-nm UV light. Figure 1 shows a representative time

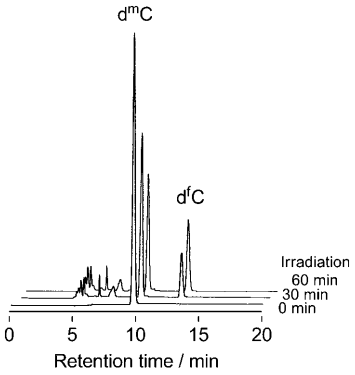


Figure 1. HPLC profiles of the photooxidation of d^{m}C (200 μM) sensitized by NQ (200 μM) upon 312-nm irradiation in 2 mM sodium cacodylate buffer solution containing 20 mM NaCl (pH 6.0) and 10% acetonitrile.

course of HPLC profiles observed in the NQ-photosensitized oxidation of d^{m}C at pH 6.0.[10] Consistent with previous reports,[11] the photoirradiation produced d^{f}C along with the degradation of d^{m}C and the corresponding HPLC peaks were assigned by reference to the respective authentic samples. In addition to the formation of d^{f}C, a few minor product peaks, which may have included 5-(hydroperoxymethyl)-2′-deoxycytidine as a reaction intermediate and 5-hydroxymethyl-2′-deoxycytidine (d^{hm}C), were observed.[12] Figure 2

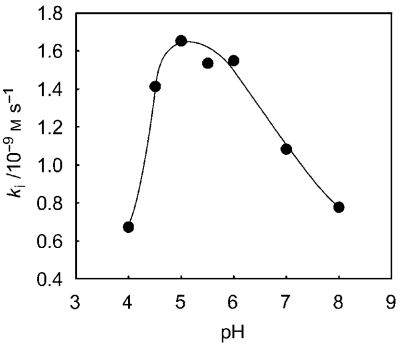
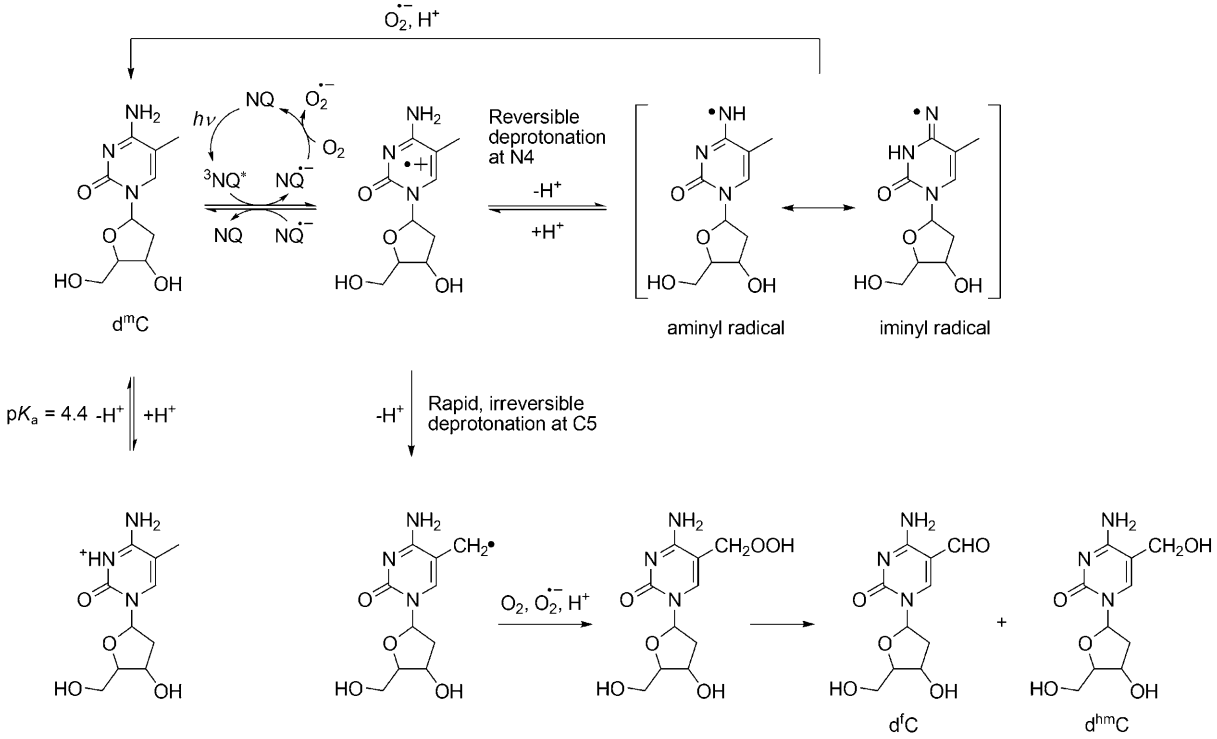


Figure 2. pH-dependent variation of the initial rate (k_i) of d^{f}C formation, as observed in the NQ-sensitized photooxidation of d^{m}C.

shows variation of the initial rate of d^{f}C formation as a function of the pH value. The initial rate of d^{f}C formation, which increased upon photoirradiation with decreasing pH values from 8.0 to 5.0, attained its maximum at pH 5.0 and then decreased dramatically at pH values below 5.0. Similar pH dependency was also observed for the rate of d^{m}C degradation during the photosensitization. These results indicate that pH value of the sample solution affected the NQ-photosensitized oxidation of d^{m}C into d^{f}C.

The photooxidation of d^{m}C by an NQ sensitizer has been studied extensively by using flash photolysis and detailed product analysis.[11,13–14] Scheme 1 shows a mechanistic outline of the photochemical conversion of d^{m}C to d^{f}C based on the results of previous studies. The initial step of oxida-



Scheme 1. A plausible reaction mechanism for the NQ-photosensitized oxidation of d^mC.

tion involves electron transfer from d^mC to the triplet-excited state NQ, thereby producing the corresponding d^mC base radical cations and NQ radical anions.[13,15] The resulting d^mC radical cation undergoes deprotonation at the methyl group to form the corresponding methyl-centered radical intermediate as shown by electron paramagnetic resonance (EPR) study,[16] followed by reaction with molecular oxygen to produce hydroperoxide that is converted to the final photooxidation products, such as d^fC and $d^{hm}C$. In competition with the deprotonation process at the methyl group, there is a protonation–deprotonation equilibrium at the exocyclic amino group on the d^mC radical cation, which is associated with regeneration of the original d^mC by reduction and subsequent protonation. Both the deprotonation at the methyl group and the protonation–deprotonation equilibrium at the exocyclic amino group of the d^mC radical cation intermediate are substantially affected by the pH change.

In a previous paper, we predicted that deprotonation of the ^mC radical cation may occur on a time-scale region comparable with the positive-charge transfer though DNA bases by reference to a proton-coupled electron-transfer process[17–18] in which electron-transfer and proton-transfer processes occur concertedly upon the one-electron redox reaction of nucleobases. This also suggests that the d^mC radical cation might favor deprotonation at the methyl group into a methyl-centered d^mC radical in the present pH range (4.0–8.0), probably owing to a much lower pK_a value ($\ll$ 4.0) for the corresponding protonation–deprotonation equilibrium. In view of the evidence that both the degradation of d^mC and formation of d^fC proceed in a pH-dependent manner, the initial oxidation and/or the reversible deprotonation–protonation process at the amino group, but not the irreversible oxygenation of methyl-centered d^mC radical, may be sensitive to the pH change ranging from 4.0 to 8.0.

To obtain a mechanistic insight into the pH effect on the initial one-electron oxidation step of d^mC, an attempt was made to measure the triplet lifetime of NQ in aqueous solution and thereby determine whether varying pH values can alter the one-electron oxidizing ability of photoexcited NQ. Upon laser flash excitation at 355 nm of NQ (50 μM) in deoxygenated phosphate buffer solution (pH 5.0–8.0), a transient absorption band assigned to the triplet NQ ($^3NQ^*$) at around 350 nm was observed,[13,15b,19–20] showing exponential decay by a self-quenching mechanism[20] (see the Supporting Information). As shown in Table 1, the triplet lifetimes of

Table 1. Triplet lifetimes (τ_T) of NQ at various pH values.[a]

pH	5.0	6.0	7.0	8.0
τ_T (μs)	1.6±0.1	1.9±0.2	1.4±0.2	1.4±0.1

[a] τ_T was determined by the first-order kinetics for the decay of absorbance at 345 nm by using 355-nm laser flash photolysis in argon-saturated 10 mM phosphate buffer solution containing 50 μM NQ.

NQ measured at various pH values were substantially similar, indicating that the chemical properties of transient $^3NQ^*$ are pH independent. This result is also supported by a previous report that the quenching of $^3NQ^*$ by thymidine was of the same rate constants at pH 3.0, 5.0, and 8.0.[13] Thus, it may be concluded that the pH change does not affect the

one-electron oxidizing ability of NQ sensitizer in the triplet excited state.

To further clarify the mechanistic details of the initial one-electron oxidation step, we also evaluated the pH dependency of the photoinduced electron-transfer reaction of d^mC by fluorescence-quenching experiments. The rate constant of electron-transfer fluorescence quenching of photoexcited organic sensitizer by d^mC was conveniently estimated by Stern–Volmer analysis. Alternative photooxidizing fluorophores of the 10-methylacridinium ion ($AcrH^+$) and 9,10-dicyanoanthracene (DCA) were employed to perform appropriate fluorescence-quenching measurements.[21] Both fluorophores have sufficient reduction potentials (2.43 and 1.95 V vs. NHE (NHE = normal hydrogen electrode) in the singlet excited state, respectively)[22] to oxidize the 5-methylcytosine base (1.73 V vs. NHE).[15] Upon excitation of $AcrH^+$ or DCA (25 μM) at 358 or 390 nm, respectively, in deoxygenated phosphate buffer solution under various pH conditions (pH 3.0–8.0), the intensity of each fluorescence emission decreased with increasing concentration of d^mC. The fluorescence-quenching rate constants (k_q) of $AcrH^{+*}$ and DCA^* were determined by the Stern–Volmer plots of the data. The k_q values evaluated at the respective pH values are plotted in Figure 3. Both k_q values of $AcrH^{+*}$ and

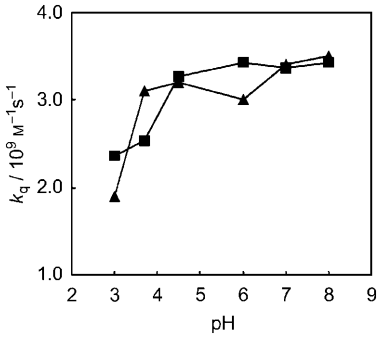
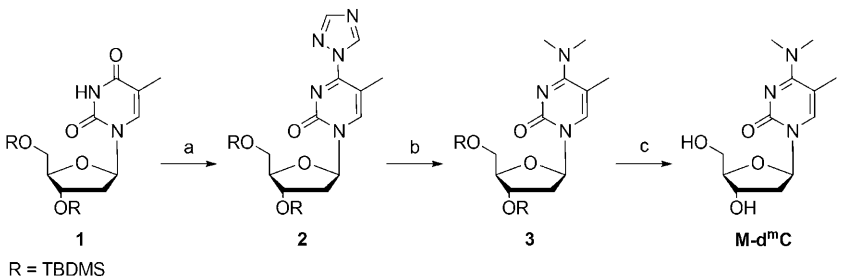


Figure 3. pH-dependent variation of the fluorescence quenching rate constant (k_q) for $AcrH^+$ (■) or DCA (▲) photosensitizing one-electron oxidation of d^mC in anoxic phosphate buffer solution (5 mM; pH 3.0–8.0).

DCA^* were fairly constant in the pH region between 5.0 and 8.0, indicating that one-electron oxidation of d^mC was not affected by the pH change in this range. In contrast, k_q values decreased drastically at pH values below 4.5. Such a decrease in the k_q value may be attributed to the increase in oxidation potential of d^mC owing to protonation at the N(3) position ($pK_a = 4.4$).[23] A similar observation was also reported for photoinduced, one-electron oxidation of cytosine 5′-monophosphate by $AcrH^+$.[22] Thus, these results clearly indicate that lower efficiencies for the degradation of d^mC and the formation of d^fC at pH values below 4.5 in the NQ-photosensitized oxidation of d^mC are attributable to the decreased rate of one-electron oxidation of d^mC that is protonated at N(3) accompanied by the increased oxidation potential.

To evaluate the effect of the reversible protonation–deprotonation equilibrium at the N(4) amino group of the d^mC radical cation on the photochemical conversion of d^mC to d^fC, we also performed the NQ-photosensitized oxidation of N(4)-dimethyl-substituted 5-methyl-2′-deoxycytidine (M-d^mC) with a lack of deprotonation at the N(4) position. M-d^mC was prepared from 3′,5′-O-bis(*tert*-butyldimethylsilyl)-thymidine **1**, as outlined in Scheme 2. Before investigating the photooxidation of M-d^mC, we measured the fluorescence quenching of DCA^* by M-d^mC to confirm that M-d^mC has an oxidation potential similar to that of the original d^mC. The k_q values of DCA at pH 7.0 in the presence of d^mC and M-d^mC were determined to be 3.4×10^9 and 4.2×10^9 $M^{-1}s^{-1}$, respectively, indicating that incorporation of the methyl group at the N4 position did not significantly decrease the oxidation potential of d^mC (see the Supporting Information). This result was also supported by the similar energy levels of the highest-occupied molecular orbitals of d^mC (−5.85 eV) and M-d^mC (−5.80 eV), as estimated by ab initio calculations at the B3LYP/6–31G* level.[24]

An aerobic aqueous solution of M-d^mC (200 μM) was photoirradiated by using 365-nm UV light[25] in the presence of NQ (200 μM) in 2 mM sodium cacodylate buffer solution containing 20 mM NaCl in the pH range between 5.0 and 8.0.[26] The pH dependency of the NQ-photosensitized degradation of M-d^mC was evaluated by HPLC (Figure 4). In contrast with the remarkable pH dependence of d^mC degradation, small changes were observed in the photosensitized degradation profile of M-d^mC with varying pH values, thus indicating that the reversible deprotonation at the N4 amino group of the d^mC radical cation seems to be responsible for the present pH-dependent NQ-photosensitized oxidation of d^mC to produce d^fC in the pH range between 5.0 and 8.0. In a separate experiment, we also examined the competitive photosensitized reaction of d^mC and M-d^mC. Similar photoirradiation at 365 nm for 10 min of an aerobic aqueous solution (pH 7.0) of d^mC and M-d^mC in the presence of NQ re-



Scheme 2. Synthesis of N(4)-dimethyl-substituted 5-methyl-2′-deoxycytidine (M-d^mC). Conditions: a) 1,2,4-triazole, $POCl_3$, Et_3N, CH_3CN, 95%; b) 50% Dimethylamine, CH_3CN, quantitative; c) TBAF $3H_2O$, THF, 87%. TBAF = tetra-*n*-butylammonium fluoride, TBDMS = *tert*-butyldimethylsilyl.

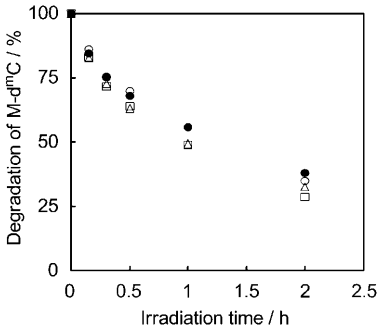
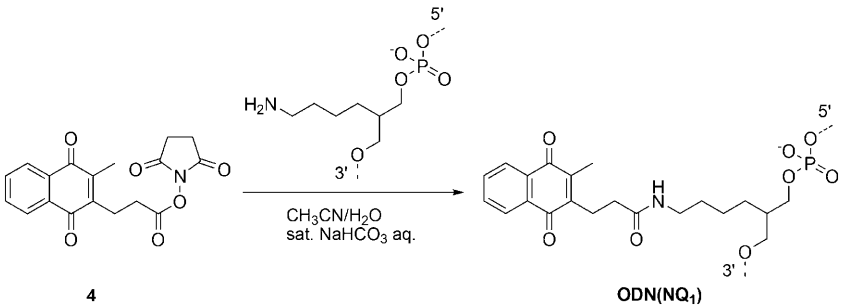


Figure 4. The pH effect on the NQ-photosensitized (200 μM) oxidative degradation of M-d^{m}C (200 μM) in aerated 2 mM sodium cacodylate buffer solution containing 20 mM NaCl and 10% acetonitrile (pH 5.0, ○; pH 6.0, □; pH 7.0, △; pH 8.0, ●).

sulted in their degradation with a conversion ratio of 9:50, despite similar oxidation potentials of these analogues (see the Supporting Information). It is therefore most likely that the d^{m}C radical cation intermediate may undergo deprotonation at the exocyclic N(4) amino group into an N(4)-amino-centered radical under neutral and even weakly acidic conditions, which suppresses an alternative deprotonation at the C(5) methyl group into a C(5)-methyl-centered radical followed by oxygenation to produce d^{f}C (see also Scheme 1). In contrast, the efficient oxygenation of the M-d^{m}C radical cation intermediate may proceed in the absence of deprotonation at the N(4)-amino group of M-d^{m}C.

Scheme 1 shows a plausible mechanism for the NQ-photosensitized oxidation of d^{m}C to form the final oxidation products. The pH effects on this oxidation can be summarized as follows: 1) In the pH range below 4.5, d^{m}C is predominantly in an N(3)-protonated form with a higher oxidation potential, therefore difficulties are encountered in undergoing one-electron oxidation to produce the corresponding radical cation intermediate. This unfavorable property of d^{m}C for one-electron oxidation accounts for decreased efficiency of the d^{f}C formation at relatively lower pH values below 4.5. 2) In the pH range between 5.0 and 8.0, d^{m}C is free from protonation at the N(3) position and so can readily undergo one-electron oxidization into the d^{m}C radical cation intermediate by NQ in the excited state, regardless of the pH conditions. Under slightly acidic conditions such as pH 5.0, the resulting d^{m}C radical cation undergoes irreversible deprotonation at the C(5) methyl group to form a methyl-centered radical intermediate, which leads to a higher yield of d^{f}C via addition of molecular oxygen to the C(5) position. Steenken reported that the radical cation of 2′-deoxycytidine is in equilibrium with its deprotonated form at the amino group even under slightly acidic conditions.[27] Thus, it is reasonable to presume that the equilibrium between the d^{m}C radical cation and its deprotonated form at the amino group may favor a shift to an increased amount of d^{m}C radical cations at pH 5.0, leading to the efficient deprotonation at the C(5) methyl group. With increasing pH values from 5.0 up to more basic conditions such as pH 8.0, the d^{m}C radical cation intermediate undergoes competitive deprotonation at the N(4)-exocyclic amino group to form an oxidizing aminyl radical[28] or its tautomer iminyl radical intermediate,[29] which may be reduced by $O_2^{-\cdot}$. These deprotonation and one-electron reduction processes may regenerate the original d^{m}C, thus resulting in a decreased yield of the photooxidation product d^{f}C. Although the pK_a value for the protonation–deprotonation equilibrium at the N(4)-exocyclic amino group of the d^{m}C radical cation has not been determined at present, it seems that the protonation–deprotonation equilibrium presumably occurs under weakly acidic conditions such as pH < 7.0 in light of the remarkable pH-dependence of d^{m}C degradation and d^{f}C formation (see Figure 2).

To characterize the photooxidative strand cleavage at the ^{m}C site in DNA, we performed the NQ-photosensitized one-electron oxidation at various pH values by using a modified ODN with the NQ chromophore in the interior of a strand (ODN(NQ$_1$)). The modified strand of ODN(NQ$_1$) was prepared by coupling of *N*-hydroxysuccinimidyl ester **4**[9a] with ODNs with various base sequences and a common aminohexyl linker (Scheme 3). In this study, we targeted the ^{m}C in a partial sequence of the human p53 gene corresponding to codons 280–285 of exon 8.[30] The sequences of ODNs used in this study are summarized in Figure 5a. Photoirradiation at 312 nm[31] of the duplex comprising ODN(NQ$_1$) and ^{32}P-5′-end-labeled ODN(^{m}C) was carried out in sodium cacodylate buffer solution (pH 4.0–8.0) containing 20 mM NaCl at 0 °C in air, and the reaction was analyzed by polyacrylamide gel electrophoresis after treatment with hot piperidine. As shown in Figure 5b, strand cleavage at ^{m}C was observed in the duplex after photoirradiation for 2 h in the pH range of 5.0–8.0, whereas only a background level of strand cleavage was observed at pH values below 4.5. As expected, the efficiency of strand cleavage at ^{m}C increased with decreasing pH, attaining a maximum efficiency at pH 5.0. These results clearly indicate that NQ-photosensitized one-electron oxidation at the ^{m}C site in DNA and subsequent formation of a methyl-centered radical occur efficiently at pH 5.0, as in the



Scheme 3. Synthesis of oligodeoxynucleotides with an NQ chromophore.

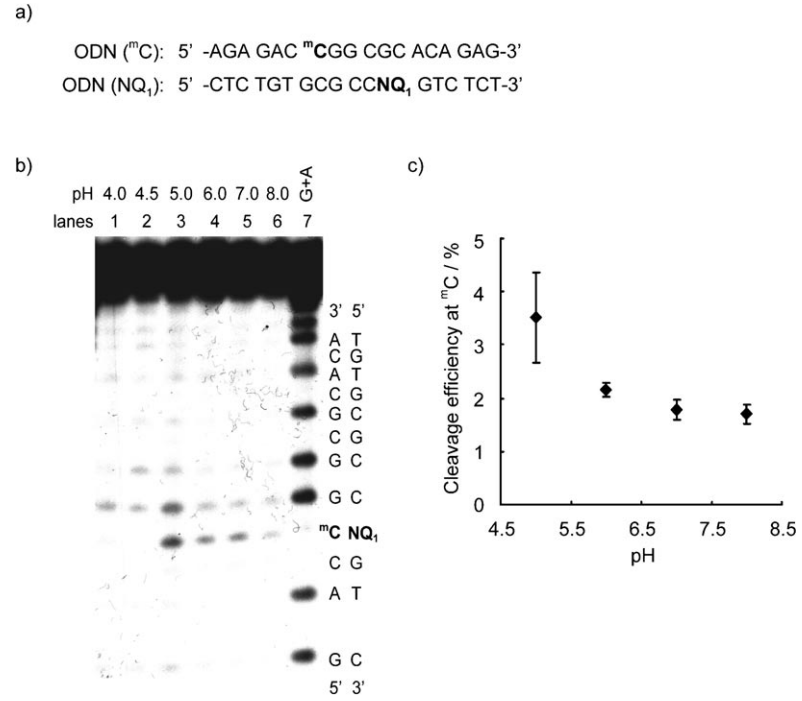


Figure 5. a) Sequences of the oligodeoxynucleotides used in this study; b) Representative autoradiogram of denaturing gel electrophoresis for ODN (NQ_1) and ^{32}P-5′-end-labeled ODN (^{m}C) upon 312 nm photoirradiation for 2 h in 2 mM sodium cacodylate buffer solution containing 20 mM NaCl (pH 4.0–8.0) at 0 °C. After treatment with hot piperidine (90 °C, 20 min), the samples were purified through electrophoresis through a denaturing 15% polyacrylamide/7 M urea: lane 1, pH 4.0; lanes 2, pH 4.5; lane 3, pH 5.0; lane 4, pH 6.0; lane 5, pH 7.0; lane 6, pH 8.0; lane 7, Maxam-Gilbert G+A sequencing lanes; c) pH-dependent efficiency of strand cleavage at the ^{m}C site estimated by densitometoric analysis of the gel image. Each error bar represents the standard deviation calculated from three sets of experimental data.

similar photooxidation of monomeric d^{m}C. Thus, the NQ-photosensitized oxidation under slightly acidic conditions is essential for strong strand cleavage at the ^{m}C site in DNA. Along with the cleavage at ^{m}C, competitive cleavage at adjacent G residues was also observed at pH 5.0 but not at pH > 6.0.[32] Douki and Cadet reported that dG is the most degradable nucleoside among four DNA bases owing to the positive-charge transfer in the NQ-sensitized photooxidation.[33] Our previous results also implied that the positive charge of the ^{m}C radical cation generated by NQ photosensitization migrates to the adjacent G base. The corresponding G radical cation is then produced in competition with the formation of a methyl-centered ^{m}C radical intermediate via deprotonation at the C(5)-methyl group.[9b] In this context, the present result suggests that the equilibrium between the ^{m}C radical cation and its deprotonated form at the amino group may favor a shift to an increased amount of ^{m}C radical cation under slightly acidic conditions such as pH 5.0, thus leading to the positive-charge transfer to a neighboring G base as a well-known positive-charge trapping site.[34,35]

Conclusion

In summary, we studied the pH effect on the NQ-photosensitized one-electron oxidation of d^{m}C. The efficiency of oxidative formation of d^{f}C from d^{m}C increased with decreasing pH from 8.0 to 5.0, attained its maximum value at pH 5.0, and then decreased dramatically upon a further decrease in pH values below 4.5. Fluorescence quenching experiments suggest that the lower efficiency for the formation of d^{f}C at pH 4.5 is attributable to the decreased rate of one-electron oxidation of d^{m}C owing to protonation at the N(3) position. The pH-independent degradation of M-d^{m}C by NQ-photosensitized oxidation indicates that the deprotonation–protonation equilibrium at the N(4)-exocyclic amino group of the d^{m}C radical cation may be a key factor in the formation of d^{f}C in the pH range between 5.0 and 8.0. We also confirmed that photosensitization of the NQ-tethered duplex at pH 5.0 allows enhanced oxidation of ^{m}C to produce 5-formylcytosine.

Although optimization of the pH environment in the photosensitized oxidation was revealed to give an efficient strand cleavage at ^{m}C in DNA, further improvements are needed to establish a standard method to analyze cytosine methylation. First, the further sensitivity for detection of ^{m}C is required. One of the key strategies for the highly sensitive detection of ^{m}C is the employment of fluorescence emission, which provides a distinct signal. We have started to develop a protocol for the fluorometric detection of ^{m}C by using a combination of photooxidative strand cleavage and invasive cleavage reaction.[9c] Second, high selectivity for the DNA cleavage at ^{m}C is indispensable. To overcome this problem, one must suppress the competitive oxidative cleavage at G caused by the positive-charge transfer in the NQ-sensitized photooxidation. Our current study focuses on the improvement of the photosensitized oxidation to give ^{m}C-specific cleavage by using other photosensitizers.

Even though the methylcytosine-selective reaction is imperative for correct detection of ^{m}C, there are few protocols for detecting ^{m}C by using methylcyotsine-positive and cytosine-negative reactions. Conventional reactions are based on cytosine-selective but not methylcytosine-selective reactions. Given these contexts, our system to directly oxidize ^{m}C but

not C could be promising for the efficient analysis of the status of cytosine methylation at a specific site in a gene.

Experimental Section

Materials: 2-Methyl-1,4-naphthoquinone was purchased from Wako Pure Chemical Industry, Japan, and was further purified by recrystallization from methanol before use. 5-Methyl-2′-deoxycytidine and 9,10-dicyanoanthracene were obtained commercially from MP Biomedicals and Tokyo Chemical Industry, respectively, and were used without further purifications. 5-Formyl-2′-deoxycytidine was prepared as described previously.[36] 10-Methylacridinium iodide ($AcrH^+I^-$) was prepared by the reactions of acridine with methyl iodide in acetonitrile,[37] and the resulting iodide salt was purified by recrystallization from 25% methanol/acetonitrile. The reagents for the DNA synthesizer were purchased from Glen Research. BD Uni-Link AminoModifier was purchased from BD Biosciences Clontech. Calf intestinal alkaline phosphatase (AP), nuclease P1 (P1), and phosphodiesterase I were purchased from PROMEGA, YAMASA, and ICN, respectively. [γ-^{32}P]ATP (6000 Ci mmol^{-1}) and T4 polynucleotide kinase (10 units μL^{-1}) were obtained from Amersham Bioscience and Nippon Gene, respectively. All aqueous solutions were prepared by using purified water (YAMATO, WR600 A).

4-(*N*-1-Triazoyl)-3′,5′-O-bis(*tert*-butyldimethylsilyl)-2′-deoxythymidine (2): 1,2,4-Triazole (6.4 g, 93 mmol) was suspended in acetonitrile (150 mL), which was cooled to 0°C and $POCl_3$ (2 mL, 21.9 mmol) was then slowly added. Triethylamine was then added dropwise and the suspension was stirred for 30 min. 3′,5′-O-Bis(*tert*-butyldimethylsilyl)thymidine (**1**; 2.0 g, 4.25 mmol) was dissolved in acetonitrile (30 mL) and added to the solution, which was then continuously stirred for another 30 min. The reaction was quenched by water and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography (SiO_2, 33% ethyl acetate–hexane) to give **2** (2.1 g, 95%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.24 (s, 1H), 8.21 (s, 1H), 8.07 (s, 1H), 6.26 (t, 1H, J = 6.3 Hz), 4.38–4.35 (1H), 4.03 (1H), 3.93 (dd, 1H, J = 11.5, 2.7 Hz), 3.77 (dd, 1H, J = 11.5, 2.4 Hz), 2.61 (ddd, 1H, J = 13.4, 6.1, 3.7 Hz), 2.41 (s, 1H), 2.04 (td, J = 12.0, 5.0 Hz), 0.88 (s, 9H), 0.86 (s, 9H), 0.09 (d, 6H, J = 5.1 Hz), 0.05 ppm (d, 6H, J = 4.9 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 158.0, 153.7, 153.3, 146.6, 145.0, 105.2, 88.7, 87.8, 62.5, 42.6, 25.9, 25.7, 18.4, 18.0, 17.2, −4.9, −5.4 ppm; FABMS (matrix: 3-nitrobenzil alcohol): m/z 522 $[M+H]^+$; HRMS: m/z calcd for $C_{24}H_{44}N_5O_4Si_2$: 522.2932 $[M+H]^+$; found: 522.2942.

4-(Dimethylamino)-3′,5′-O-bis(*tert*-butyldimethylsilyl)-2′-deoxythymidine (3): 50% Dimethylamine in aqueous solution (8 mL) was added to a solution of **2** (1.0 g, 1.92 mmol) in acetonitrile (20 mL) and the mixture was stirred at 0°C for 5 min. The reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography (SiO_2, 33% hexane–ethyl acetate) to give **3** (1.0 g, quantitative) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.40 (s, 1H), 6.27 (t, 1H, J = 6.5 Hz), 4.31–4.27 (1H), 3.84–3.66 (3H), 3.06 (s, 6H), 2.31 (ddd, 1H, J = 13.3, 6.1, 3.7 Hz), 2.06 (s, 3H), 1.96–1.87 (1H), 0.85 (s, 9H), 0.81 (s, 9H), 0.03 (d, 6H, J = 1.3 Hz), −0.02 ppm (d, 6H, J = 1.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 165.9, 155.0, 140.2, 102.1, 87.3, 85.4, 71.5, 62.6, 41.8, 40.1, 25.8, 25.6, 18.3, 17.9, −4.7,-5.5 ppm; FABMS (matrix: 3-nitrobenzyl alcohol): m/z 498 $[M+H]^+$; HRMS: calcd for $C_{24}H_{48}N_3O_4Si_2$: 498.3183 $[M+H]^+$; found: 498.3192.

4-(Dimethylamino)-2′-deoxythymidine (M-d^{m}C): Tetrabutylammonium fluoride trihydrate (0.94 g, 3.0 mmol) was added to a solution of **3** (0.5 g, 1.0 mmol) in dry THF (10 mL) and the mixture was stirred at room temperature for 20 min. The reaction mixture was then concentrated and the crude residue was resuspended with water and then washed with chloroform. The aqueous layer was concentrated in vacuo. The crude product was roughly purified by column chromatography (SiO_2, 0–10% methanol-chloroform) and then purified by reversed-phase HPLC (15% acetonitrile–water) to give M-d^{m}C (234 mg, 87%) as a white solid. mp. 182–183°C; ^{1}H NMR (D_2O, 400 MHz): δ = 7.41 (s, 1H), 6.13 (t, 1H, J = 6.6 Hz), 4.32 (m, 1H), 3.90 (m, 1H), 3.68 (ddd, 2H, J = 31.5, 12.3, 4.9 Hz), 3.07 (s, 6H), 2.27–2.12 (2H), 2.09 ppm (s, 3H); ^{13}C NMR ([D_6]DMSO, 100 MHz): δ = 164.9, 153.7, 141.0, 101.8, 87.2, 84.6, 70.3, 61.2, 40.2, 39.8, 18.1 ppm; FABMS (matrix: 3-nitrobenzyl alcohol): m/z 270 $[M+H]^+$; HRMS: calcd for $C_{12}H_{20}N_3O_4$: 270.1453 $[M+H]^+$; found: 270.1454.

Photosensitized oxidation by 2-methyl-1,4-naphthoquinone: Solutions of 5-methyl-2′-deoxycytidine (200 μM) or N(4)-dimethyl-substituted 5-methyl-2′-deoxycytidine (200 μM) in 2 mM sodium cacodylate buffer solution (various pH values) containing 20 mM NaCl were added to an acetonitrile solution of 2-methyl-1,4-naphthoquione (200 μM). The solutions (100 μL) in a 1.5-mL Eppendorf tube were exposed to 312 nm UV light with a Lourmat TFX-20 M transilluminator (Vilber Lourmat, France) or 365 nm UV light with a TFL-40X transilluminator (UVP, USA) at 0°C. Analytical HPLC was performed with a Shimadzu 6 A HPLC system. Sample solutions (10 μL) were injected onto a reversed-phase column (Inertsil ODS-3, GL Sciences Inc., φ 4.6 mm × 150 mm). The solvent mixture of triethylamine acetate (0.1 M, pH 7.0) containing various concentrations of acetonitrile (5 or 15 vol%) was delivered as the mobile phase. The column eluents were monitored by the UV absorbance at 260 nm.

Nanosecond laser flash photolysis: The laser-flash-photolysis experiments were carried out with a Unisoku TSP-601 flash spectrometer. A Continuum Surelite-I Nd:YAG (Q-swiched) laser with the third harmonic at 355 nm (approximately 50 mJ per 6-ns pulse) was employed for the flash photoirradiation. Further details of the laser flash system have been described previously.[38] Solutions of NQ (50 μM) in 10 mM phosphate buffer solution (pH 5–8) were de-aerated by passing argon through the solution prior to the laser flash photolysis experiments.

Fluorescence quenching: Quenching experiments of the fluorescence of photosensitizers were carried out on a Shimadzu RF-5300PC spectrophotometer. The excitation wavelengths were 358 and 390 nm for 10-methylacridinium ion (25 μM) and 9,10-dicyanoanthracene (25 μM), respectively. The monitoring wavelengths were those corresponding to the respective emission bands at 453 and 487 nm, respectively. The dynamic quenching rate constant k_q was determined by the Stern–Volmer equation: $I_0/I = 1 + k_q\tau_0$[5-methyl-2′-deoxycytidine], where I_0/I is the ratio of the emission intensities in the absence and presence of 5-methyl-2′-deoxycytidine and τ_0 is the lifetime of the singlet excited state of 10-methylacridinium ion (35 ns)[37] or 9,10-dicyanoanthracene (15.1 ns)[39] in the absence of quencher.

Synthesis of ODNs: Synthesis of oligodeoxynucleotides was performed on an Applied Biosystems Model 392 DNA/RNA synthesizer by using standard phosphoroamidite chemistry techniques. We used BD Uni-Link AminoModifier for DNA synthesis to introduce an aminohexyl group into oligomers. After automated DNA synthesis, the oligomer was purified by reversed-phase HPLC with a 0–30% linear gradient (over 60 min) of acetonitrile/0.1 M TEAA buffer solution (TEAA = triethylammonium acetate) at pH 7.0. The purity and concentration of the oligomer were determined by complete digestion with AP, P1, and phosphodiesterase I at 37°C for 4 h. The synthesized oligomers were identified by MALDI-TOF mass spectrometry (negative mode: calcd. 5312.47, found 5312.05). A solution of 3-(*N*-hydroxysuccinimidylethyl)-2-methyl-1,4-naphthoquinone[9a] (68 μg, 0.2 μmol) and saturated $NaHCO_3$ (20 μL) was added to a solution (total volume 50 μL) of oligomer with an amino linker in the interior of the strand and subsequently incubated at 25°C for 4 h. The reaction mixture was purified by reversed-phase HPLC with a 0–30% linear gradient (over 60 min) of acetonitrile/0.1 M TEAA buffer solution at pH 7.0. The purity and concentration of NQ-modified ODN were determined by complete digestion by AP, P1, and phosphodiesterase I at 37°C for 4 h. The synthesized ODNs were identified by MALDI-TOF mass spectrometry (ODN (NQ_1); m/z: calcd for 5538.69 $[M-H]^-$; found: 5539.59).

Preparation of 5′-^{32}P-end labeled ODNs: ODNs (400 pmol strand concentration) were labeled by phosphorylation with 4 μL of [γ-^{32}P]ATP and 46 μL of T4 polynucleotide kinase by using standard procedures.[40–41] The 5′-end-labeled ODNs were recovered by ethanol precipitation and fur-

ther purified by 15% nondenaturing gel electrophoresis and isolated by the crush-and-soak method.[42]

Photooxidative cleavage of ODNs: ^{32}P-5′-end labeled ODNs (<400 nM strand concentration) were hybridized by their complementary ODNs with NQ chromophore (500 nM) in 2 mM sodium cacodylate buffer solution (pH 7.0) containing 20 mM NaCl. Hybridization was achieved by heating the sample at 90°C for 5 min and slowly cooling to room temperature. 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 the addition of 10 μL of herring-sperm DNA or salmon-sperm DNA (1 mg mL^{-1}), 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 disolved in 50 μL of 10% piperidine (v/v), heated at 90°C for 20 min and concentrated. The resulting samples were then analyzed by polyacrylamide gel electrophoresis, of which the experimental details were described previously.[9]

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$$\Delta G_{CS} = -(E_T + E_{rdn}) + E_{ox} \quad (1)$$

where E_T is the NQ triplet energy[15b] (2.52 eV), E_{rdn} is its reduction potential[15c] (−0.21 V vs. NHE), and E_{ox} is the oxidation potential of 5-methylcytosine (1.73 V vs. NHE) as calculated from the ionization potential[15d] (8.39 eV).[15e] See: a) D. Rehm, A. Weller, *Isr. J. Chem.* **1970**, *8*, 259–271; b) T. Melvin, E. Bothe, D. Schulte-Frohlinde, *Photochem. Photobiol.* **1996**, *64*, 769–776; c) P. Wardman, *J. Phys. Chem. Ref. Data* **1989**, *18*, 1637–1755; d) C. J. Burrows, J. G. Muller, *Chem. Rev.* **1998**, *98*, 1109–1151; e) D. M. Close, *J. Phys. Chem. B* **2003**, *107*, 864–867.

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[26] The pK_a value for M-d^{m}C was determined to be 3.8 from the pH-response of the absorbance at 260 nm, indicating that the effect of the N(3) protonation of M-d^{m}C should be negligible in the pH range above 5.0 (see the Supporting Information).

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