Highly selective detection of 5-methylcytosine using photochemical ligation†

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We report the nonenzymatic detection of 5-methylcytosine by using template-directed photoligation through 5-cyanovinyl-2′-deoxyuridine (^{C}U) with high selectivity and present a new methylation detection method using a photoligation-based DNA chip assay.

DNA methylation patterns are a cell epigenetic program that controls the expression of genetic information.[1] Epigenetics is the study of heritable codes other than genetic codes written in A, G, C and T. Therefore, cells or individuals with the same genetic information can have different phenotypes. Monozygotic twins and cloned animals are examples of individuals with the same genetic information and different phenotypes. DNA methylation and histone modifications (acetylation and methylation) serve as epigenetic code. Analysis of DNA methylation is one of the most intensely developing fields of molecular biology. DNA methylation is critical for normal embryo development. Many studies testify to substantial changes in the DNA methylation pattern in tumors. There have been reports on cytosine methylation analysis based on chemical concepts and enzymatic methods; for example, hydrolysis and sequencing with a bisulfite salt,[2] bipyridine ligands for direct 5-methylcytosine labeling and their application to fluorescent and electrochemical assay,[3] and cleavage assay with methylation-insensitive restriction enzymes,[4] and another chemical approach.[5] Although the conventional methods have many merits, there are many disadvantages, and methylation detection assays must be further improved through another approach. We recently demonstrated a method for site-specific transition of 5-methylcytosine to a thymine analog *via* reversible DNA photoligation.[6] The DNA template-directed reversible photoligation proceeded *via* [2 + 2] cycloaddition between the double bond of the 5-carboxyvinyl-2′-deoxyuridine side chain and the C5–C6 double bond of pyrimidine.[7] Photochemical ligation has the benefit of not needing additional reagents. Additionally, we previously demonstrated photochemical end-capping of oligodeoxynucleotides *via* 5-cyanovinyl-2′-deoxyuridine (^{C}U) analogs.[8] Here, we focus on the properties of ^{C}U, and report on the control of the 5-methylcytosine selective addition on DNA using photochemical ligation *via* a ^{C}U (Fig. 1).

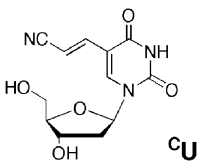

Fig. 1 Structure of 5-cyanovinyl-2′-deoxyuridine.

Our strategy is based on stacking stability induced by hydrophobic interaction between the presence/absence of a methyl group at the C5 position of the cytosine and the cyanovinyl group of the uridine derivative. In the presence of the methyl group, we expected photoligation to be promoted by effective stacking between the cyanovinyl group and the C5–C6 double bond (Fig. 2). In contrast to the absence of the methyl group, photocrosslinking is promoted by the intercalation manner of the cyanovinyl group and the opposite site bases of the template DNA. In this paper, we explain that photoligation is the connection of photosensitive DNA to the natural DNA strand on the template DNA, and photocrosslinking is the connection of photosensitive DNA to the template DNA.

The modified ODN was prepared, according to standard phosphoramidite chemistry, on a DNA synthesizer. Incorporation of ^{C}U ODN1(^{C}U) into the ODN was confirmed by enzymatic digestion and MALDI-TOF MS (calcd. 1860.34 for ODN1(^{C}U) [$(M + H)^{+}$], found 1860.73). The ODNs used in this study are summarized in Table 1. We determined the

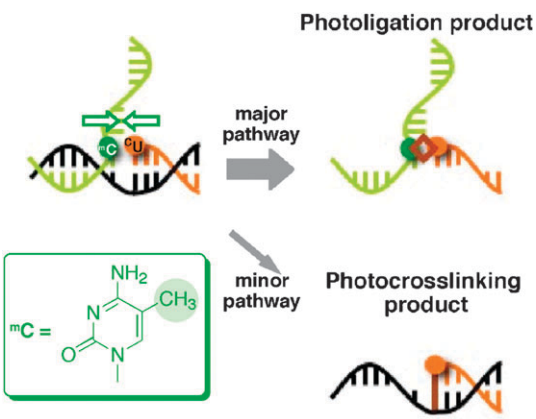

Fig. 2 Strategy for 5-methylcytosine detecting method *via* photosensitive DNA.

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Table 1 ODN sequences used in this study

	Sequences (5′–3′)[ab]
ODN1(^{C}U)	^{C}UGCGTG
ODN1(^{m}C)	GAGAG^{m}CAAAAA
cODN1	CACGCAGCTCTC
ODN2(^{C}U)	^{C}UGACGTGTATCGCATTGGSSSSNH$_2$
ODN2(C)	GCCCCAGCTGCTCACCATCGCTATCTGA GCAGCGC TCATGGTGGGGGCAGCGCCTCACAACCT CCGTCAT GTGCTGTGACTGCTTGTAGATGGCCATGGC
ODN2(^{m}C)	GCCCCAGCTGCTCACCATCGCTATCTGAGC AGCGCTCATGGTGGGGGCAG^{m}CGCCTCACA ACCTCCGTCATGTGCTGTGACTGCTTGTAGA TGGCCATGGC
cODN2	CGATACACGTCAGCTGCCCCCACCA
ODN(Cy3)	Cy3-CGCTGCTCAGATAGC

[a] Underlined characters indicate a methylation status. [b] S corresponds to a hexa(ethylene glycol) linker fragment.

feasibility of photoligation of ^{m}C-containing ODN.[9] When ODN1(^{m}C) and ODN1(^{C}U) were irradiated at 366 nm for 15 min in the presence of template cODN1, we observed the concomitant reduction of ODN1(^{m}C) and ODN1(^{C}U) and the appearance of ODN1(^{m}C–^{C}U) as a new peak at a retention time of 21 min by HPLC analysis (Fig. 3). MALDI-TOF MS indicated that isolated ODN1(^{m}C–^{C}U) obtained from HPLC purification was a ligated product of ODN1(^{m}C) and ODN1-(^{C}U) (calcd. 5282.56 for ODN1(^{m}C–^{C}U) [(M + H)$^{+}$], found 5282.57). And then, enzymatic digestion of isolated ODN1(^{m}C–^{C}U) showed the formation of dC, dG, T and dA in a ratio of 1 : 6 : 1 : 7, together with a d(^{m}C–^{C}U) photoadduct (calcd. 520.192 for d(^{m}C–^{C}U) [(M + H)$^{+}$], found 520.334). In order to estimate the ratio of the ligated product to the crosslinking product (Scheme 1), we calculated the ratio of

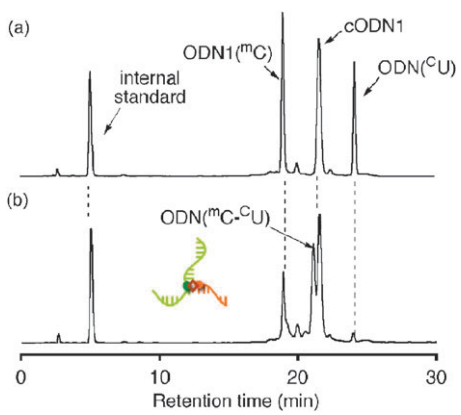
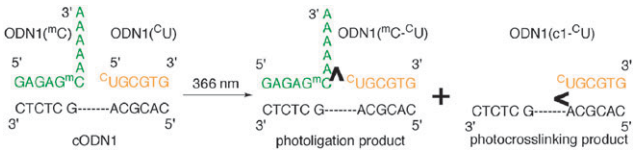


Fig. 3 HPLC analysis of ODN1(^{C}U) and ODN1(^{m}C) in the presence of template cODN1 irradiation at 366 nm. (a) Before photoirradiation. (b) Irradiation at 366 nm for 15 min. 2′-Deoxycytidine (dC) was used as an internal standard.



Scheme 1 Template-directed DNA photoligation of ODNs with ^{C}U.

d(^{m}C–^{C}U) photoadduct to the d(A-^{C}U) photoadduct corresponded to the crosslinking product from the HPLC profile of enzymatic digestion (see ESI†).[10] The yield of ligated product was 5.5-fold higher than that of crosslinking product in the case of ODN1(^{m}C). In contrast to using the ODN1(^{m}C), the yield of crosslinking product was 1.6-fold higher than that of ligated product in the cytosine case (see ESI†). From the above-mentioned results, we established that the ratio of the ligated ODN to the crosslinking ODN in the 5-methylcytosine case was 9.1-fold higher than that in the cytosine case.

To demonstrate that template-directed photoligation by using ODN2(^{C}U) could be incorporated into platforms suitable for DNA chip technologies, we constructed a DNA chip by attaching amino-labeled ODN containing ^{C}U onto the aldehyde-modified glass surface. We determined the feasibility of the template-directed photoligation through ODN2(^{C}U) on a DNA chip. We prepared 60 mer DNA strands, 5′-d(...GGGGGCAG**X**GCCTCACAACC...)-3′, which contained a methylation hotspot (**X** = ^{m}C or C) at codon 175 in exon 5 of the p53 gene.[11] A glass chip spotted with 2 μM target ODN2(C) or ODN2(^{m}C) and template cODN2 was irradiated at 366 nm for 30 min in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride. After the chip had been washed with deionized water at 98 °C for 5 min, Cy3-containing ODN(Cy3) conjugate was added to the surface, and the chip was washed twice in PBS. Fluorescence signals were detected on a microarray scanner. As shown in Fig. 4A, we measured the strong fluorescence signal of the photoligated product with the ^{m}C case. The results show that a ^{m}C-containing ODN2(^{m}C) yielded highly photoligated product, with a measured fluorescence signal that was 9.8-fold higher than the C-containing ODN2(C) case (Fig. 4B). Additionally, we estimated the effect of the cyano group by using 5-vinyl-2′-deoxyuridine (^{V}U) instead of ^{C}U. We used the same method to measure the ratio of photoligation to photocrosslinking yield by using ^{V}U (see ESI†). However, we observed no meaningful difference in the case of using ^{V}U. This result suggests that hydrophobicity was clearly changed by the presence/absence of the cyano group. We considered that the

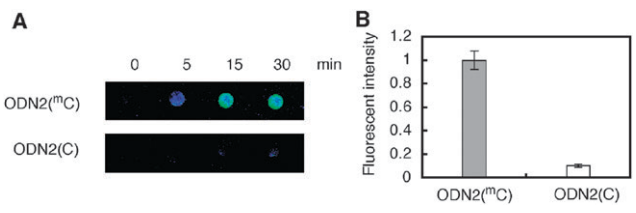
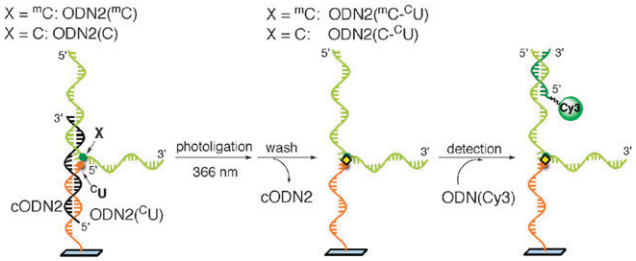


Fig. 4 (A) Fluorescence images acquired on a microarray scanner for the product of photoligation on cytosine and 5-methylcytosine target DNAs. (B) Selectivity for different photosensitive nucleotide conditions. The data points represent the average of three experimental runs by irradiation at 366 nm for 15 min.



Scheme 2 Conceptual scheme showing how the target is detected by photoligation.

photoreactive cyanovinyl group in ^{C}U was effectively stacked with the C5–C6 double bond of 5-methylcytosine by the methyl group at the C5 position, which stabilizes the hydrophobic interaction.[12] However, ^{C}U was stacked with base in the opposite strand adenine in the absence of the methyl group at the cytosine, which allows stabilization in an intercalation manner. As a result, photocrosslinking ODN was synthesized by using cytosine. The direction of photoreaction may be applicable for detecting the presence/absence of the methyl group at cytosine C5. We previously reported that the photochemical DNA end-capping *via* ODN containing photosensitive pyrimidine at the 5′-terminal was changed by the micro-environment such as the hydrophobic interaction and intercalation manner in the DNA strands.[8]

In conclusion, we have demonstrated a 5-methylcytosine detecting system by using a photoligation method thorough ^{C}U. Significantly, 5-methylcytosine in the target sequence yielded ligated product, with a measured ratio of ligation yield that was 9.8-fold higher than the cytosine case. The photochemical ligation approach includes many advantages such as not needing additional reagents. This new protocol (Scheme 2) can detect 5-methylcytosine for high-throughput analysis of DNA methylation.

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9 The reaction mixture (total volume 100 μl) containing ODN1(^{C}U) (20 μM, strand conc.), ODN1(^{m}C) (20 μM, strand conc.) and cODN1 (22 μM, strand conc.) in 50 mM sodium cacodylate buffer (pH 7.0) and sodium chloride was irradiated with a UV-LED (366 nm ± 15 nm light at 1600 mW cm^{-2}) at a distance of 1.5 cm at 0 °C for 15 min. After irradiation, the progress of photoreaction was monitored by HPLC. The yield was calculated on the basis of ODN1(^{m}C). The reaction mixture was taken up and subjected to HPLC analysis. Analysis was carried out on a COSMOSIL 5C$_{18}$-AR-II column (4.6 × 150 mm), with detection at 260 nm; elution was with 0.05 M ammonium formate containing 3–20% acetonitrile, linear gradient (30 min) at a flow rate of 1.0 ml min^{-1}, 30 °C.

10 The reaction mixture (total volume 30 μl) containing ODN1(^{C}U) (20 μM, strand conc.), ODN1(^{m}C) (20 μM, strand conc.) and cODN1 (22 μM, strand conc.) in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM sodium chloride was irradiated with a UV-LED (366 nm ± 15 nm light at 1600 mW cm^{-2}) at a distance of 1.5 cm at 0 °C for 15 min. After irradiation, the reaction mixture was subjected to enzymatic digestion with s. v. PDE (0.25 unit mL^{-1}), P-1 nuclease (2.5 unit mL^{-1}) and AP (10 unit mL^{-1}) for 4 h to decompose it to mononucleosides. The reaction mixture was taken up and subjected to HPLC analysis. Analysis was carried out on a COSMOSIL 5C$_{18}$-AR-II column (4.6 × 150 mm), with detection at 260 nm; elution was with 0.05 M ammonium formate containing 3–20% acetonitrile, linear gradient (30 min) at a flow rate of 1.0 ml min^{-1}, 30 °C.

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