Photochemical Ligation of DNA Probe prepared in Click Chemistry

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Template-directed photoligation with 5-carboxyvinyl-2'-deoxyuridine (^{CV}U) can be used for genomic research, biotechnology and biomedical agents. However, in photoligation with ^{CV}U, a long photoirradiation time at 366 nm is required to complete photoligation. To overcome the limitation of photoligation with ^{CV}U, we synthesized new photosensitive probes in the copper-catalyzed azide-alkyne cycloaddition reaction, as the best example of click chemistry.

Keyword: DNA, photoligation, click chemistry

1. Introduction

DNA ligation methods are important techniques for genomic research, biotechnology and biomedical agents.[1–3] In our previous work, we reported on the template-directed reversible photoligation with 5-carboxyvinyl-2'-deoxyuridine (^{CV}U) derivative used as a form of phototriggered DNA manipulation.[4–6] The advantage of using photons as reagents to initiate photoligation is that no additional chemical reagents need to be introduced into the system. However, in photoligation with ^{CV}U, a long photoirradiation time at 366 nm is required to complete photoligation. To overcome the limitation of photoligation with ^{CV}U, we tried to synthesize new photosensitive probes in click reaction. The copper-catalyzed azide-alkyne cycloaddition reaction, the best example of click chemistry, appears to fulfill the necessity. The reaction was discovered by the groups of Meldal and Sharpless.[7,8] It functions efficiently in aqueous media and produces a 1,2,3-triazole linkage between the two reactants. We hypothesized that the electron-donating properties of the triazole ring might effectively enhance photoligation. And

within the field of nucleic acid chemistry, Cu(I)-catalyzed azide-alkyne cycloaddition has been used for labeling oligonucleotides with a range of moieties, such as fluorescein. Thus, we also attempted to synthesize the DNA probe for quick single nucleotide polymorphism (SNP)

Scheme 1. Schematic illustration of photoligation of photoresponsive oligonucleotides (ODNs).

typing using photochemical ligation, as an example.

2. Experimental

2.1 General method and materials

¹H NMR spectra were measured with Varian Gemini 300 (300 MHz) spectrometer. ¹³C NMR spectra were measured with Varian INOVA 500 (125 MHz) spectrometer. Coupling constant (*J* value) are reported in hertz. The chemical shifts are reported in δ (ppm) relative to residual CDCl₃ (δ = 7.24 in ¹H NMR), CD₃OD (δ = 3.35 in ¹H NMR, δ = 49.3 in ¹³C-NMR) and DMSO-*d*₆ (δ = 2.49 in ¹H NMR, δ = 39.7 in ¹³C NMR) as internal standards. Mass spectra were recorded on a Voyager-DE PRO-SF, Applied Biosystems. HPLC was performed on a Chemcobond 5-ODS-H column (10 × 150 mm, 4.6 × 150 mm) or a Chemcosorb 5-ODS-H column (4.6 × 150 mm) with a JASCO PU-2080 plus, MX-2080-32, DG-2080-53 system equipped with a JASCO UV 2075 detector at 260 nm. The reagents for the DNA synthesizer such as A, G, C, T- β -cyanoethyl phosphoramidite, and CPG support were purchased from Glen Research. JASCO V-550 UV/VIS spectrophotometer was used for absorption spectra measurements. Photoirradiation was performed by UV-LED (OMURON, 366 nm) or 25 W transilluminator (FUNAKOSHI, TFL-40, 366 nm, 5,700 μ W/cm²).

2.2 (*E*)-5-(2-Carbomethoxyvinyl)-2'-deoxyuridine (2)

5-Iodo-deoxyuridine (1.00 g, 2.82 mmol) and Pd(OAc)₂ (0.06 g, 0.28 mmol) were suspended in DMF (3 mL) in a 10 mL glass vial equipped with a small magnetic stirring bar. To this was added the Bu₃N (0.67 mL, 2.82 mmol) and methylacrylate (0.38 mL, 4.24 mmol) and the vial was tightly sealed with an aluminum/Teflon® crimp top. The mixture was then irradiated for four minutes at 100 °C, using an irradiation power of 60 W. After completion of the reaction, the vial was cooled to 50 °C by gas jet cooling before it was opened. It was then filtered and evaporated *in vacuo*. The crude was purified by column chromatography (CHCl₃ / MeOH = 9 / 1) to give **2** (0.84 g, 95%). ¹H NMR (DMSO-*d*₆, 300 MHz) δ : 11.63 (bs, 1H), 8.40 (s, 1H), 7.36 (d, 1H, *J* = 15.5

Hz), 6.84 (d, 1H, *J* = 15.5 Hz), 6.12 (t, 1H, *J* = 6.3 Hz), 5.24 (d, 1H, *J* = 4.3 Hz), 5.15 (t, 1H, *J* = 5.3 Hz), 4.24 (m, 1H), 3.79 (m, 1H), 3.67 (s, 3H), 3.64-3.54 (m, 2H), 2.17 (m, 2H).

2.3 (*E*)-5-(2-Carboxyvinyl)-2'-deoxyuridine (3)

2 (8.52 g, 27.29 mmol) was added to 3M NaOH (water/ ethanol = 1 / 1, 10 ml) and this mixture stirred at room temperature for 3 h. HCl was powered into this solution on cooling ice bath to give white precipitate. The precipitate was filtered and washed with hexane, and sucked dry. The product was dried under vacuum to yield **3** (7.24 g, 89%). ¹H NMR (DMSO-*d*₆, 300 MHz) δ : 12.16 (bs, 1H), 11.62 (s, 1H), 8.37 (s, 1H), 7.28 (d, 1H, *J* = 15.9 Hz), 6.76 (d, 1H, *J* = 15.9 Hz), 6.12 (t, 1H, *J* = 6.2 Hz), 5.25 (d, 1H, *J* = 4.1 Hz), 5.18 (t, 1H, *J* = 5.1 Hz), 4.24 (m, 1H), 3.79 (m, 1H), 3.60 (m, 2H), 2.16 (m, 2H).

2.4 (*E*)-5-(2-Bromovinyl)-2'-deoxyuridine (4)

To a solution of **3** (5.00 g, 23.5 mmol) in DMF (100 ml) was added K₂CO₃ (4.87 g, 35.2 mmol) at ambient temperature and stirred for 15 minutes. A solution of *N*-Bromosuccinimide (4.18 g, 23.5 mmol) in DMF (100 ml) was added dropwise over 30 min. The resulting suspension was filtered immediately under suction and the solid was washed well with DMF. The combined filtrate and washing were evaporated to dryness in vacuo to complete remove the DMF and the crude was purified by column chromatography (CHCl₃ / MeOH = 9 / 1) to give **4** (3.94 g, 50%) ¹H NMR (DMSO-*d*₆, 300 MHz) δ : 11.56 (s, 1H), 8.06 (s, 1H), 7.23 (d, 1H, *J* = 13.6 Hz), 6.83 (d, 1H, *J* = 13.6 Hz), 6.11 (t, 1H, *J* = 6.6 Hz), 5.25 (d, 1H, *J* = 4.4 Hz), 5.09 (t, 1H, *J* = 5.2 Hz), 4.23 (m, 1H), 3.77 (m, 1H), 3.57 (m, 2H), 2.12 (m, 2H).

2.5 (*E*)-5-(2-trimethylsilyl ethynyl vinyl)-2'-deoxyuridine (5)

A mixture of **4** (3.35 g, 10.1 mmol), Pd(PPh₃)₄ (1.17 g, 1.01 mmol), CuI (0.382 g, 2.01 mmol) and *N,N*-diisopropylethylamine (8.56 ml, 50.4 mmol) in DMF (50 ml) was stirred for 10 minutes. To this there was then added trimethylsilylacetylene (4.18 ml, 30.2 mmol) and the mixture was stirred for 2 h. The resulting suspension was then filtered and the crude was

purified by column chromatography (CHCl_3 / MeOH = 9 / 1) to give **5** (2.38 g, 67%) ^1H NMR (CD_3OD , 300 MHz) δ : 8.29 (s, 1H), 6.71 (d, 1H, J = 16.1 Hz), 6.64 (d, 1H, J = 16.1 Hz), 6.30 (t, 1H, J = 6.5 Hz), 4.45 (m, 1H), 3.97 (m, 1H), 3.84 (m, 2H), 2.32 (m, 2H), 0.21 (m, 9H); ^{13}C NMR (CD_3OD , 125 MHz) δ : 164.2, 151.5, 141.0, 136.0, 112.3, 109.9, 106.5, 96.7, 89.4, 87.2, 72.1, 62.8, 42.1.

2.6 5'-O-(4,4'-Dimethoxytrityl)-(E)-5-(2-trimethylsilyl ethynyl vinyl)-2'-deoxyuridine (**6**)

To a solution of **5** (0.60 g, 1.7 mmol) in anhydrous pyridine (10 ml) was added DMTrCl (0.70g, 2.0 mmol) and DMAP (0.04 g, 0.34 mmol) at 0°C. The reaction mixture was stirred for 4 h at ambient temperature. The reaction mixture was evaporated to dryness under reduced pressure. The crude product was purified by silica gel column chromatography (CHCl_3 / MeOH = 97 / 3) to afford **6** (0.34 g, 31%). ^1H NMR (CDCl_3 , 300MHz) δ : 8.61 (bs, 1H), 8.60 (bs, 1H), 7.39-7.24 (m, 9H), 6.83 (m, 4H), 6.58 (d, 1H, J = 16.1 Hz), 6.30 (m, 1H), 5.98 (d, 1H, J = 16.1 Hz), 4.51 (m, 1H), 4.08 (m, 1H), 3.78 (s, 6H), 3.74 (s, 1H), 3.40 (d, 2H, J = 3.3 Hz), 2.46 (m, 1H), 2.26 (m, 1H), 0.16 (m, 9H); ^{13}C NMR (CD_3OD , 125 MHz) δ : 162.0, 158.4, 158.3, 149.4, 145.0, 140.4, 136.1, 135.6, 135.5, 130.0, 129.9, 128.1, 127.9, 127.0, 113.4, 110.0, 107.6, 105.7, 96.0, 85.9, 85.8, 84.7, 70.3, 64.0, 56.3, 55.2, 0.1.

2.7 5'-O-(4,4'-Dimethoxytrityl)-(E)-5-(2-trimethylsilyl ethynyl vinyl)-2'-deoxyuridine phosphoramidite (**7**)

To a solution of **6** (0.20 g, 0.31 mmol) in dry CH_3CN (5 ml) in a sealed bottle was added 0.45 M tetrazole in CH_3CN (0.68 ml, 0.31 mmol) and 2-cyanoethyl -*N,N,N',N'*-tetraisopropylphosphorodiamidite (0.097 ml, 0.31 mmol) and the reaction mixture was stirred for 2 h at ambient temperature. The reaction mixture was diluted with EtOAc and organic layer was washed with a saturated aqueous solution of NaHCO_3 . The organic layer was collected, dried over anhydrous sodium sulfate, and evaporated to dryness to yield **7**, which was directly used in an automated DNA synthesizer without further purification.

2.8 Preparation of ODN($^{\text{EV}}$ U)

ODN sequences were synthesized by the conventional phosphoramidite method by using an Applied Biosystems 3400 DNA synthesizer. The coupling efficiency was monitored with a trityl monitor. The coupling efficiency of crude mixture of $^{\text{EV}}$ U was 97% yield. The coupling time of crude mixture of $^{\text{EV}}$ U was 999 sec. They were deprotected by incubation with 28% ammonia for 4 h at 65 °C and were purified on a Chemcobond 5-ODS-H column (10 × 150 mm) by reverse phase HPLC; elution was with 0.05 M ammonium formate containing 3-20% CH_3CN , linear gradient (30 min) at a flow rate of 3.0 mL/min. Preparation of oligonucleotides was confirmed by MALDI-TOF-MS analysis.[9]

2.9 Huisgen cycloaddition between ODN($^{\text{EV}}$ U) and azides

A reaction mixture (total volume 2.0 mL) containing ODN($^{\text{EV}}$ U) (50 μM strand conc.), a solution of an azide in EtOH (2.5 mM), a aqueous solution of CuSO_4 (2 mM) and a aqueous solution of sodium ascorbate (1 mM) was shaken for 12 h. The crude product was purified on a Chemcobond 5-ODS-H column (10 × 150 mm) by reverse phase HPLC; elution was with 0.05 M ammonium formate containing 3-20% CH_3CN , linear gradient (30 min) at a flow rate of 3.0 mL/min.

2.10 Template-directed photoligation

A reaction mixture (total volume 200 μL) containing ODN(T) (10 μM strand conc.), photoligation probe (10 μM strand conc.) and ODN(6A) (12 μM strand conc.) in 50 mM sodium cacodylate buffer (pH 7.0) was irradiated at 0 °C with UV-LED (366 nm). After irradiation 10 μL of the aliquot was taken up and subjected to HPLC analysis. Analysis was carried out on a Chemcobond 5-ODS-H column (4.6 × 150 mm), detected at 260 nm; elution was with 0.05 M ammonium formate containing 5–8% acetonitrile, linear gradient (20 min), then 8–30% acetonitrile, linear gradient (20 min) at a flow rate of 1.0 mL/min. Irradiation at 366 nm caused the ligated product. They were characterized by MALDI-TOF-MS [10].

3. Results and Discussion

Scheme 2. Reagents and conditions: (a) $\text{Pd}(\text{OAc})_2$, Bu_3N , methylacrylate, DMF, $100\text{ }^\circ\text{C}$, 4 min, 95%; (b) NaOH , water/ethanol = 1/1, rt, 3 h, 89%; (c) K_2CO_3 , *N*-bromosuccinimide, DMF, rt, 30 min, 50%; (d) $\text{Pd}(\text{PPh}_3)_4$, CuI , *N,N*-diisopropylethylamine, trimethylsilylacetylene, rt, 2 h, 67%; (e) 4,4'-dimethoxytrityl chloride, DMAP, pyridine, rt, 4 h, 31%; (f) $(i\text{Pr}_2\text{N})_2\text{PO}(\text{CH}_2)_2\text{CN}$, 1*H*-tetrazole, CH_3CN , rt, 2 h, quant.; (g) DNA synthesizer.

Scheme 3. Synthesis of photoresponsive ODNs.

The nucleoside phosphoramidite of 5-ethynylvinyl-2'-deoxyuridine ($^{\text{EVU}}$) was

prepared (Scheme 2), and this monomer was incorporated into ODN using standard automated DNA synthesis protocols. After deprotection and purification of the oligomer, containing $^{\text{EVU}}$, four azides were purchased and synthesized according to a method reported in the literature[11]. Huisgen cycloaddition between $\text{ODN}(^{\text{EVU}})$ and azides was carried out. After purification of the products, photoresponsive ODNs (Scheme 3) were characterized by MALDI-TOF-MS.[10]

$\text{ODN}(^{\text{CVU}})$	5'- $^{\text{CVU}}$ UGCGTG-3'
$\text{ODN}(^{\text{BTVU}})$	5'- $^{\text{BTVU}}$ UGCGTG-3'
$\text{ODN}(^{\text{PTVU}})$	5'- $^{\text{PTVU}}$ UGCGTG-3'
$\text{ODN}(^{\text{MPTVU}})$	5'- $^{\text{MPTVU}}$ UGCGTG-3'
$\text{ODN}(^{\text{NTVU}})$	5'- $^{\text{NTVU}}$ UGCGTG-3'
$\text{ODN}(^{\text{BNTVU}})$	5'- $^{\text{BNTVU}}$ UTCAGTGTA-3'
$\text{ODN}(\text{T})$	5'-TGTGCT-3'
$\text{ODN}(\text{6A})$	5'-CACGCAAGCACA-3'
Capture ODN	5'- NH_2 -SSSS-ACTCACAGTTTTTACAC-3'
$\text{ODN}(\text{WT})$	5'-GGAGTGGCCGGGAGTTGGGCGAGTACGGGCTGCAGGCATACACT GAAGTGA AAACTGTGAGTGTGGGACCTGCTGGGGCTCAGGGCCTGTTGGGGCTTG-3'
$\text{ODN}(\text{MUT})$	5'-GGAGTGGCCGGGAGTTGGGCGAGTACGGGCTGCAGGCATACACT AAAGTGA AAACTGTGAGTGTGGGACCTGCTGGGGCTCAGGGCCTGTTGGGGCTTG-3'

Figure 1. ODNs used in this study. Bold bases indicate the SNP site.

Figure 2. HPLC analysis of photoligation with $\text{ODN}(^{\text{NTVU}})$ and $\text{ODN}(\text{T})$ in the presence of $\text{ODN}(\text{6A})$.

We determined the feasibility of photoligation of the ODNs containing a triazole ring and ODN(T) in the presence of ODN(6A). It is also shown that the photoligation rates with ODNs containing a triazole ring are more rapid than the photoligation rates with ODN(^{CV}U). The time in which the photoligation rates reached the 42% conversion in 5 sec (Figure 3). Photoligation on the second time scale becomes possible with NTVU.

Figure 3. Photoligation yield obtained by photoirradiation at 366 nm for 5 s by using photoresponsive ODNs.

Figure 4. Comparison of photoligation rates with ODN(^{NTV}U). Open circle symbols represent ODN(T) that had been photoligated, and filled square symbols denote ODN(C) that had been photoligated.

There was a difference between the photoligation rates by using ODN(^{CV}U) with 3'-terminal T and the photoligation rates by using ODN(^{CV}U) with 3'-terminal C. Thus, it was

necessary to change handling procedures depending on the target of photoligation. But, there was little difference between the photoligation rates by using ODN(^{NTV}U) with 3'-terminal T and the photoligation rates by using ODN(^{NTV}U) with 3'-terminal C. Thus, it is not necessary to change handling according to depending on the target of photoligation. Therefore photoligation by using ODN(^{NTV}U) is very simple and easy.

Figure 5. Strategy and fluorescence images for the detection of single nucleotide differences on a DNA chip.

To show that this new photosensitive ODN probe is suitable for practical use, we performed a quick SNP typing as an example. We synthesized naphthyl azide containing biotin, and Huisgen cycloaddition between the azide and ODN (^{EV}U) was carried out. After purification of the products, ODN (^{BNTV}U) was characterized by MALDI-TOF-MS.[12] We constructed the DNA chip by attaching amino-labelled ODN (capture ODN), onto the aldehyde-modified glass surface. A glass chip spotted with 2 mM target, ODN (WT) or ODN (MUT) and ODN (^{BNTV}U) was irradiated at 366 nm for 10 min in 50 mM sodium cacodylate buffer (pH 7.0) (Figure 5). After the chip had been washed with deionized water at 98°C for 5 min, a phosphate-buffered saline (PBS) solution of strept-avidin-Cy3 conjugate was added to the surface, and the chip was washed twice in PBS. Fluorescence signals were detected on a micro array scanner. As shown in Figure 5, we measured the strong fluorescence signal of the photoligated

product with the completely complementary case, showing a measured rate that was 10^3 -fold higher than that of the case with a single mismatch in the target sequence.

4. Conclusion

In summary, we performed the rapid and efficient synthesis of new photosensitive probes in the copper-catalyzed azide-alkyne cycloaddition reaction. Photoligation with these probes proceeds very quickly (second time scale). We also performed quick SNP typing with a photochemical probe in which biotin was incorporated in click chemistry. The reactivity of these probes will be beneficial to in situ DNA manipulation.

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