Fluorescence labeling of DNA based on photochemical ligation

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Abstract

Fluorescent labeling of oligonucleotides has been attracting interest in connection with the development of methods for distinguishing and detecting nucleic acids sequences. And photochemical ligation has the merit of the avoiding the need for additional reagents. Fujimoto et al. reported template directed DNA photoligation using 5-carboxyvinyl-deoxyuridine (CVU). Here, we describe the synthesis and photocrosslinking ability of fluorescent reporter analogue (Cy5) tethered CVU-containing ODN.

Keywords: Fluorescent labeling of oligonucleotides; Photochemical ligation; Photocrosslinking

1. Introduction

Fluorescent labeling of oligonucleotides has been attracting current interest in connection with the development of new methods for distinguishing and detecting nucleic acids sequences for diagnostics and homogeneous hybridization assays. Being dependent on the local environment fluorescence has proved an indispensable tool for the study of molecular interactions and several cellular functions. Fluorescently labeled oligonucleotide probes are nowadays in much regular use for nucleic acid sequencing [1], sequencing by hybridization (SBH) [2], fluorescence in situ hybridization (FISH) [3], fluorescence resonance energy transfer (FRET) [4], molecular beacons [5,6], taqman probes [7]. This has made fluorescent probes an important tool for clinical diagnostics and made possible real-time monitoring of oligonucleotide hybridization. Chemical approaches involving cross-linking reactions have been quiet useful [8,9].

Photochemical ligation has the merit of the avoiding the need for additional reagents. Their actions are controllable within space and time by the choice of proper irradiation methods. Thus, the photoligation methods can be used as ‘photopadlocking’ of circular DNA, as a tool for DNA engineering and nanotechnology, and as photoregulated diagnostic and therapeutic agents [10–13]. And branched DNA molecules have various uses in signal amplification technology [14], nanotechnology applications such as DNA computing [15], DNA nanostructures using self-assembled branched units [16], DNA sensors [17], and nanoelectronic devices [18].

Fujimoto et al. reported template directed DNA photoligation using 5-carboxyvinyl-deoxyuridine (CVU). CVU-containing ODN have high photoreactivity at 366 nm irradiation. And the resulting ligated DNA is quantitatively reverted to the original oligonucleotides by 302 nm irradiation. By using this novel photoligation method, a convergent and versatile synthesis of branched ODN that would be particularly useful in DNA nanotechnology is possible [12].

Here, we describe the synthesis and photocrosslinking ability of fluorescent reporter analogue (Cy5) tethered CVU-containing ODN.

2. Experimental

2.1. General method and materials

Dioxane, pyridine, DMTrCl, was purchased from Kanto Chemical. 5-Iodo-2’-deoxyuridine was purchased from Tokyo Kasei. Ethyl trifluoroacetate, palladium (II) acetate, PPh3, methyl acrylate, 2-cyanoethyl N,N,N’,N’-tetra-isopropyl-phosphoramidite and N,hydroxy succinimide were purchased from Aldrich. DMAP was purchased from ACROS.
ORGANICS. 1H-tetrazole was purchased from Glen Research. Ethylenediamine was purchased from Nacalai tesque. 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride salt was purchased from Sigma. The reagents for the DNA synthesizer such as I2 solution (I2/H2O/pyridine/tetrahydrofuran, 3:2:19:76), A-, G-, C-, and T-β-cyanoethyl phosphoramidites were purchased from Glen Research. Other reagents were purchased at highest commercial quality and used without further purification unless otherwise stated. Calf intestine alkaline phosphatase (AP) (1500 units) was purchased from Roche. Nuclease P1 (500 units) was purchased from Sigma. The reagents for the DNA phosphoroamidites were purchased from Glen Research. Hydrofuran, 3:2:19:76), A-, G-, C-, and T-deoxyuridine as a white powder. 1H NMR (CDCl3) δ 8.31 (s, 1H, H-6), 7.41–7.38 (m, 7H), 7.06 (d, 1H, vinyl, J = 15.8 Hz), 6.13 (t, 1H, H-1', J = 6.5 Hz), 5.25 (d, 1H, 3'-OH, J = 4.2 Hz), 5.16 (t, 1H, 5'-OH, J = 5.1 Hz), 4.24 (m, 1H, H-3'), 3.79 (m, 1H, H-4'), 3.67 (s, 3H, OCH3), 3.64–3.54 (m, 2H, H-5'), 2.17 (m, 2H, H-2').

2.2.3. (E)-5-(2-carboxyvinyl)-2'-deoxyuridine (5)

To a solution of 5 (0.79 g, 2.65 mmol) in anhydrous pyridine (12 ml) was added DMAP (0.03 g, 0.33 mmol) at ambient temperature. To a solution of DMTc (0.99 g, 2.91 mmol) in anhydrous pyridine (8 ml) was added at 0°C. The reaction mixture was stirred for 4 h at ambient temperature. TCL analysis showed the absence of starting material. The reaction mixture was evaporated to dryness under reduced pressure. The crude product was purified by silica gel column chromatography (CHCl3:MeOH = 97:3, v/v) to afford 6 (0.68 g, 43%) as white solid. 1H NMR (CDCl3) δ 7.93 (s, 1H, H-6), 7.40–6.70 (m, 15H, vinyl, Ar-H), 6.13 (t, 1H, H-1', J = 6.5 Hz), 4.37 (br, 1H, 3'-OH), 4.01 (m, 2H, H-3', H-4'), 3.67 (s, 6H, OCH3), 3.40 (m, 2H, H-5'), 2.37–2.27 (m, 2H, H-2').

2.2.4. 5'-O-(4,4'-dimethoxytrityl)-(E)-5-(2-carboxymethylyvinyl)-2'-deoxyuridine (6)

To a solution of 6 (0.30 g, 0.50 mmol) in dry CH2CN (20 ml) was added N-hydroxysuccinimide (0.075 g, 0.60 mmol) and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride salt (0.114 g, 0.60 mmol) cooling by ice bath and the solution was stirred for 18 h at ambient temperature. The reaction mixture was stirred for 6 h. The precipitate was removed by filtration, and evaporated. The residue was extracted with chloroform (20 ml × 3) and water (30 ml), and washed with brine (20 ml × 2). The organic layer was collected, dried over anhydrous magnesium sulphate, and evaporated to dryness under reduced pressure. The crude product was purified by column chromatography (ethyl acetate, ethyl acetate) to afford 7 as a white solid. 1H NMR (CDCl3) δ 8.13 (s, 1H, NH), 7.94 (s, 1H, H-6), 7.41–7.38 (m, 2H), 7.30–7.18 (m, 7H), 7.06 (d, 1H, vinyl, J = 15.5 Hz), 6.83–6.80 (m, 4H), 6.64 (d, 1H, vinyl, J = 15.5 Hz), 6.28 (t, 1H, J = 6.6 Hz, 1'-H), 5.55 (bs, 1H, 3'-OH), 4.49 (m, 1H, 3', H-2'), 4.07 (m, 1H, 4'-H), 3.75 (d, 6H, OCH3), 3.47 (m, 2H, 5', H-2'), 3.33–3.30 (m, 4H), 2.27 (m, 2H, 2', H-2'), MALDI-TOF MS: calculated for C37H32F3N4O7Na[(M+Na)+] 761.2410, found 761.2574.

2.2.2. (E)-5-(2-carboxyvinyl)-2'-deoxyuridine (4)

Palladium (II) acetate (0.15 g, 0.9 mmol), triphenylphosphine (0.37 g, 1.9 mmol), and triethylamine (2.5 ml, 18 mmol) were combined in anhydrous dioxane (25 ml) and stirred at 75°C until an intense red developed. 5-Iodo-2-deoxyuridine (5.0 g, 14 mmol) and methyl acrylate (2.35 ml, 27 mmol) were then added, and refluxed at 115°C under nitrogen atmosphere for 1 h. TLC of the reaction mixture in ethyl acetate) to afford 5-carbomethoxyvinyl-2-deoxyuridine (4).1H NMR (CDCl3) δ 3.37 (br, 2H), 2.90 (t, 2H, J = 5.9 Hz).

2.2.2. (E)-5-(2-carboxyvinyl)-2'-deoxyuridine (4)

(E)-5-(2-carboxyvinyl)-2'-deoxyuridine (4) was obtained by condensation of 5-(E)-5-(2-carboxyvinyl)-2'-deoxyuridine with ethylenediamine. 1H NMR (CDCl3) δ 7.93 (s, 1H, H-6), 7.40–6.70 (m, 15H, vinyl, Ar-H), 6.13 (t, 1H, H-1', J = 6.5 Hz), 4.37 (br, 1H, 3'-OH), 4.01 (m, 2H, H-3', H-4'), 3.67 (s, 6H, OCH3), 3.40 (m, 2H, H-5'), 2.37–2.27 (m, 2H, H-2').

2.2.5. 5'-O-(4,4'-dimethoxytrityl)-(E)-5-(2-carboxymethylyvinyl)-2'-deoxyuridine (7)

To a solution of 6 (0.30 g, 0.50 mmol) in dry CH2CN (20 ml) was added N-hydroxysuccinimide (0.075 g, 0.60 mmol) and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride salt (0.114 g, 0.60 mmol) cooling by ice bath and the solution was stirred for 18 h at ambient temperature, then 2 (0.078 g, 0.50 mmol) was added and stirred for 6 h. The precipitate was removed by filtration, and evaporated. The residue was extracted with chloroform (20 ml × 3) and water (30 ml), and washed with brine (20 ml × 2). The organic layer was collected, dried over anhydrous magnesium sulphate, and evaporated to dryness under reduced pressure. The crude product was purified by column chromatography (ethyl acetate) to afford 7 as a white solid. 1H NMR (CDCl3) δ 8.13 (s, 1H, NH), 7.94 (s, 1H, H-6), 7.41–7.38 (m, 2H), 7.30–7.18 (m, 7H), 7.06 (d, 1H, vinyl, J = 15.5 Hz), 6.83–6.80 (m, 4H), 6.64 (d, 1H, vinyl, J = 15.5 Hz), 6.28 (t, 1H, J = 6.6 Hz, 1'-H), 5.55 (bs, 1H, 3'-OH), 4.49 (m, 1H, 3', H-2'), 4.07 (m, 1H, 4'-H), 3.75 (d, 6H, OCH3), 3.47 (m, 2H, 5', H-2'), 3.33–3.30 (m, 4H), 2.27 (m, 2H, 2', H-2'), MALDI-TOF MS: calculated for C37H32F3N4O7Na[(M+Na)+] 761.2410, found 761.2574.
2.2.6. 5''-O-(4,4''-dimethoxytrityl)-3''-O-[2-cyanoethoxy-(N,N-diisopropylamino)-phosphino]-5-(E)-(N-[2-(2,2,2-trifluoroacetylamino)-ethyl]-acylamide)-2''-deoxyuridine (8)

To a solution of 7 (0.24 g, 0.32 mmol) in dry CH₃CN (5 ml) in a sealed bottle was added 0.45 M tetrazole in CH₃CN (0.71 ml, 0.32 mmol) and 2-cyanoethyl N,N,N',N'-tetra-isopropylphosphoramidite (0.10 ml, 0.32 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 NaHCO₃. The organic layer was collected, dried over anhydrous sodium sulfate, and evaporated to dryness to yield 8, which was directly used in an automated DNA synthesizer without further purification.

2.3. Synthesis of amine containing oligonucleotides (ODN 1)

Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramide method on a controlled pore glass supports by using Applied Biosystems Model 3400 synthesizer. The 0.1 M acetonitrile solution of 8 was used in automated synthesis, the oligonucleotide were cleaved from the support by conc. aqueous ammonia for 1 h, deprotected by heating the solutions at 55 °C for 8 h, and purified by reverse phase HPLC. The purity and concentration of all nucleotides were determined by digestion with AP, Nuclease P1 to 2'-deoxymononucleotide at 37 °C for 3 h. MALDI-TOF MS: calcd for ODN 1 [(M+H)⁺] 1919.3804, found 1919.3847.

Scheme 1. Reagents and conditions: (a) ethyl trifluoroacetate (1.0 equiv.), −78 °C, (b) methylacrylate (2.0 equiv.), Pd(OAc)₂ (0.05 equiv.), PPh₃ (0.15 equiv.)/dioxane, reflux 115 °C, 2 h, (c) 3 M NaOH, rt 3 h, then 6 M HCl, 0 °C, (d) DMAP (0.1 equiv.), DMTrCl (1.1 equiv.)/pyridine, 18 h, (e) EDAC (1.2 equiv.), NHS (1.2 equiv.), 2 (1.0 equiv.), (f) [(iPr)₂N]₂POCH₂CH₂CN (1.0 equiv.), 1H-tetrazole (1.0 equiv.)/acetonitrile, (g) DNA synthesizer, (h) Cy5-NHS (1.0 equiv.)/100 mM tetraborate buffer.
2.4. Synthesis of Cy5 containing oligonucleotides (ODN 2)

To a solution of ODN 1 (0.25 μmol) in 100 mM tetraborate buffer (pH 8.5) was added Cy5-NHS (0.2 mg, 0.25 mM) and the reaction mixture was incubated for 2 h at ambient temperature. The crude was purified by reverse phase HPLC to afford ODN 2 (0.11 μmol, 42%). The purity and concentration of all nucleotides were determined by digestion with AP, Nuclease P1 to 2'-deoxymononucleotide at 37 °C for 3 h. MALDI-TOF MS: calcd for ODN 2 [M+H] 2559.6075, found 2559.6084.

2.5. Photoligation of DNA oligomer as monitored by reverse phase HPLC

The reaction mixture (total volume 300 µl) containing ODN 2 (20 nM, strand conc.), ODN 3 (20 nM, strand conc.), ODN 4 (25 nM, strand conc.) in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM NaCl in a Pyrex tube was irradiated at 0 °C with a 25 W transilluminator (366 nm, 5,700 μW cm⁻²) under otherwise identical conditions. After irradiation, 5 µL of aliquot was taken up and subjected to reverse phase HPLC analysis (elution with a solvent mixture of 50 mM ammonium formate, pH 7.0, linear gradient over 15 min from 6 to 9% acetonitrile then over 15 min from 9 to 40% acetonitrile at a flow rate of 0.8 ml min⁻¹). Photoligated product was separated and identified by MALDI-TOF-MS. The purified ligated product was enzymatically digested with Nuclease P1 and AP at 37 °C for 4 h to 2'-deoxymononucleosides and photoligated dimmer (HPLC condition; 0.05 M ammonium formate containing 3–20% acetonitrile, linear gradient (30 min) at a flow rate of 1.0 ml/min).

2.6. Synthesis of branched ODN as monitored by reverse phase HPLC

The reaction mixture (total volume 300 µl) containing ODN 2 (20 nM, strand conc.), ODN 6 (20 nM, strand conc.), ODN 4 (25 nM, strand conc.) in 50 mM sodium cacodylate buffer (pH 7.0) and 100 mM NaCl in a Pyrex tube was irradiated at 0 °C with transilluminator (366 nm) under otherwise identical conditions. After irradiation, 5 µL of aliquot was taken up and subjected to reverse phase HPLC analysis (elution was with 0.05 M ammonium formate containing 6–12% acetonitrile, linear gradient (30 min) at a flow rate of 1.0 ml min⁻¹). Photoligated product was separated and identified by MALDI-TOF-MS. The purified ligated product was enzymatically digested with Nuclease P1 and AP at 37 °C for 4 h to 2'-deoxymononucleosides and photoligated dimmer (HPLC condition; 0.05 M ammonium formate containing 3–20% acetonitrile, linear gradient (30 min) at a flow rate of 1.0 ml/min).

2.7. Measurement of fluorescent spectrum

Fluorescent spectrum of ODN 7 was obtained using JASCO FP-6500 spectrofluorometer at room temperature using 5 mm path length cell. The excitation bandwidth was 1 nm. The emission bandwidth was 1 nm.

3. Result and discussion

3.1. Synthesis

One of the amino groups of ethylenediamine was protected by trifluoroacetate group to yield 2. 5-Iododeoxyuridine 3 was transferred to 4 by the Heck reaction. Methyl ester group of 4 was hydrolyzed then dimethoxytritylated and coupled with 2 by condensation reaction to yield 7. Compound 7 was converted to the corresponding cyanoethyl phosphoramidite using a conventional method. The CVU with amino group-containing ODN (ODN 1) was synthesized on an ABI3400 DNA synthesizer. Succinimidy ester of Cy5 was coupled with ODN 1 to yield Cy5 and CVU-containing ODN (ODN 2) (Scheme 1).
A formation of ODN 2 was confirmed by enzymatic digestion and MALDI-TOF MS.

3.2. Photoligation of DNA oligomer

We determined the feasibility of photoligation of the Cy5 tethered CVU-containing ODN (Scheme 2). ODN 2 and 5'-d(TGTGCC)-3' (ODN 3) were irradiated at 366 nm for 180 min at 0 °C in the presence of template ODN 4. HPLC analysis of a mixture of ODN 2 and ODN 3 photoirradiated with template ODN 4 indicated a clean and efficient formation of ligated ODN 5 and the concomitant disappearance of ODN 2 and ODN 3 (Figs. 1 and 2). Enzymatic digestion of isolated ODN 5 showed the formation of dC, dG, and dT in a ratio of 2:5:3, together with a new product. The molecular weight of ODN 5 was equal to the sum of the molecular weight of ODN 2 and ODN 3. It is strongly suggested that the photoligation reaction proceeded via [2+2] cycloaddition between the double bond of CVU side chain and the C5–C6 double bond of cytosine, giving rise to the formation of a cyclobutane structure as observed for CVU without Cy5 [11,12].

3.3. Synthesis of branched ODN

We next tried the synthesis of branched ODN (Scheme 3). ODN 2 and 5'-d(TGTGCCAAAAA)-3' (ODN 6) were irradiated at 366 nm for 180 min at 0 °C in the presence of template ODN 4. HPLC analysis of a mixture of ODN 2 and ODN 6 photoirradiated with template ODN 4 indicated a clean and efficient formation of ligated ODN 7 and the concomitant disappearance of ODN 2 and ODN 6 (Figs. 3 and 4). Enzymatic digestion of isolated ODN 7 showed the formation of dC, dG, and dT in a ratio of 2:5:3, together with a new product. The molecular weight of ODN 7 was equal to the sum of the molecular weight of ODN 2 and ODN 6. It is strongly suggested that Synthesis of branched ODN succeeded via [2+2] cycloaddition.

3.4. Fluorescent spectrum

Fluorescent spectrum of ODN 7 was measure (Fig. 5). A strong fluorescence at 663 nm was observed on excitation at 635 nm, characteristic of Cy5. This results shows that Cy5-labeled ODN was successfully synthesized.
4. Conclusion

In conclusion, we synthesized Cy5 tethered CVU-containing ODN. We determined the feasibility of photoligation of the Cy5 tethered CVU-containing ODN. We succeeded the synthesis of branched and Cy5-labeled ODN. These results indicate that method provides the new site-specific labeling method of diagnostic sensing of nucleic acid sequences.

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References

[1] H.A. Erlich, D. Gelfond, J.J. Srinsky, Recent advances in the polymerase chain reaction, Science 252 (1991) 1643–1651.
[2] A.D. Mirzabekov, DNA sequencing by hybridization—a megasequencing method and a diagnostic tool? TIBTECH 12 (1994) 27–32.
[3] E.J.M. Speel, A.H.N. Hopman, P. Komminoth, Amplification methods to increase the sensitivity of in situ hybridization: play CARD(s), J. Histochem. Cytochem. 47 (1999) 281–288.
[4] P.R. Selvein, The renaissance of fluorescence resonance energy transfer, Nat. Struct. Biol. 7 (2000) 730–734.
[5] N.E. Broude, Stem-loop oligonucleotides: a robust tool for molecular biology and biotechnology, TIBTECH 20 (2002) 249–256.
[6] http://www.molecular-beacons.org
[7] C.T. Wittwer, M.G. Herrmann, A.A. Moss, R.P. Rasmussen, Continuous fluorescence monitoring of rapid cycle DNA amplification, Biotechniques 22 (1997) 130–139.
[8] O. Kornyushyna, A.J. Stemmler, D.M. Graybosch, I. Bergenthal, C.J. Burrows, Synthesis of a metallopeptide-PNA conjugate and its oxidative cross-linking to a DNA target, Bioconjugate Chem. 16 (2005) 178–183.
[9] G.F. Ross, P.M. Smith, A. McGregor, D.M. Turnbull, R.N. Lightowlers, Synthesis of trifunctional PNA-benzophenone derivatives for mitochondrial targeting, selective DNA binding, and photo-cross-linking, Bioconjugate Chem. 14 (2003) 962–966.
[10] J. Liu, J.S. Taylor, Template-directed photoligation of oligodeoxyribonucleotides via 4-thiothymidine, Nucleic Acids Res. 26 (1998) 3300–3304.
[11] K. Fujimoto, S. Matsuda, N. Takahashi, I. Saito, Template directed photoreversible ligation of deoxyoligonucleotides via 5-vinyldeoxyuridine, J. Am. Chem. Soc. 122 (2000) 3564–3567.
[12] K. Fujimoto, N. Ogawa, M. Hayashi, S. Matsuda, I. Saito, Template directed photochemical synthesis of branched oligodeoxyribonucleotides via 5-carboxyvinyldeoxyuridine, Tetrahedron Lett. 41 (2000) 9437–9440.
[13] (a) Kenzo Fujimoto, Yoshinaga Yoshimura, Tadayoshi Ikemoto, Akio Nakazawa, Masayuki Hayashi, Isao Saito, Photoinduced DNA end capping via N1-methyl-5-cyano-2-deoxyuridine, Chem. Commun. 25 (2005) 3177–3179;
(b) M. Ogino, Y. Yoshimura, A. Nakazawa, I. Saito, K. Fujimoto, Template-directed DNA photoligation via a -5-cyano-2-vinyldeoxyuridine, Org. Lett. 7 (2005) 2853–2856;
(c) S. Ogasawara, K. Fujimoto, Solution of a SAT problem on a photochemical DNA computer, Chem. Lett. 34 (2005) 378–379;
(d) Y. Yoshimura, Y. Ito, K. Fujimoto, Interstrand photocrosslinking of DNA via p-carbamoylvinyl phenol nucleoside, Bioorg. Med. Chem. Lett. 15 (2005) 1299–1301.
[14] (a) M.S. Urdea, Branched DNA signal amplification, Bio/Technology 12 (1994) 926–928;
(b) M.L. Collins, B. Irvine, D. Tyner, E. Fine, C. Zayati, C. Chang, T. Horn, D. Ahle, J. Detmer, L.P. Shen, J. Kolberg, S. Bushnell, M.S. Urdea, D.D. Ho, A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/m, Nucleic Acids Res. 25 (1997) 2979–2984.
[15] P. Aldyen, N. Jonoska, N.C. Seeman, Self-assembly of irregular graphs whose edges are DNA helix axes, J. Am. Chem. Soc. 126 (2004) 6648–6657.
[16] M. Scheffler, A. Dorenbeck, S. Jordan, G. Wustefeld, G. Kiedrowski, Self-assembly of trisoligonucleotides: the case for nano-acetylene and nano-cyclobutadiene, Angew. Chem. Int. Ed. 38 (1999) 3311–3315.
[17] F. Nakamura, E. Ito, Y. Sakou, N. Ueno, I.N. Gatuna, F.S. Ohuchi, M. Hara, Preparation of a branched DNA self-assembled monolayer toward sensitive DNA biosensors, Nano Lett. 3 (2003) 1083–1086.
[18] H.A. Becerril, R.M. Stoltenberg, D.R. Wheeler, R.C. Davis, J.N. Hard, A.T. Woolley, DNA-templated three-branched nanostructures for nanoelectronic devices, J. Am. Chem. Soc. 127 (2005) 2828–2829.
[19] The yield was calculated based on ODN 3.
[20] The yield was calculated based on ODN 6.

Fig. 5. Fluorescent spectrum of ODN 7. Excitation wavelength was 635 nm. \(\lambda_{\text{em}} = 663\, \text{nm}\).