Fluorescence labeling of DNA based on photochemical ligation

Takehiro Ami[1] and Kenzo Fujimoto[1, 2,*]

[1]The School of Material Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Isikawa 923-1292, Japan

[2]PRESTO, Japan Science and Technology Agency (JST) Kawaguch 332-0012, Japan

[*] Corresponding author.

E-mail: kenzo@jaist.ac.jp

Tel: +81-761-51-1673

Fax: +81-761-51-1671

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 (^{CV}U). Here we describe the synthesis and photocrosslinking ability of fluorescent reporter analogue (Cy5) tethered ^{CV}U-containing ODN.

Keyword

fluorescent labeling of oligonucleotides, photochemical ligation, photocrosslinking

Main text

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 (^{CV}U). ^{cv}U-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 ^{cv}U-containing ODN.

2. Experimental

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, PPh_3, methyl acrylate, 2-cyanoethyl *N*,*N*,*N'*,*N'*-tetra-isopropyl- phosphorodiamidite and *N'*-hydroxysuccinimide were purchased from Aldrich. DMAP was purchased from ACROS ORGANICS. 1*H*-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 I_2 solution (I_2/ H_2O/ pyridine/tetrahydrofuran, 3: 2: 19: 76), A-, G-, C-, and T-β-cyanoethyl phosphoroamidites were purchased from Glen Research. Other regents were purchased at highest commercial quality and used without further purification unless otherwise stated. Calf intestine alkaline phosphstase (AP) (1500 units) was purchased from Roche. Nuclease P1 (500 units) was purchased from Yamasa. Reactions were monitored TLC plates precoated with Merck silica gel 60 F_{254}. Kanto Chemical Silica Gel 60 N was used for silica gel column chromatography. ^{1}H-NMR spectrum

was recorded on Varian Gemini-300 (300 MHz). Coupling constant (*J* value) are reported in hertz. The chemical shift are reported in ẟ(ppm) relative to residual chloroform (δ = 7.24) and DMSO (δ = 2.49) as internal standards. ODNs were synthesized on an Aplid Biosystems 3400 DNA Synthesizer. Reverse phase HPLC was performed on a Chemcobond 5C18 ODS column (4.6 x 150 mm) with a JASCO PU-2080, MX-2080-32, DG-2080-53 system equipped with a JASCO UV 2075 spectrometer at 260 nm. Takara PCR Thermal Cycler MP was used for heating of oligonucleotides. Mass spectra were recorded on a Voyager-DE PRO-SF, Applied Biosystems.

2.2. Synthesis of mononucleosides

*2.2.1. N-(2-amino-ethyl)-2,2,2-trifluoro-acetamide (**2**)*

Ethylenediamine (**1**) (5 ml, 74.71 mmol) was dissolved in MeOH (50 ml) and cooled to –78 °C. A solution of ethyltriuoroacetate (CF_3COEt) (8.89 ml, 74.71 mmol) was added drop wise to the diamine and the reaction temperature was maintained for 1.5 h. After warming to 0 °C over 1 h, the MeOH solvent was evaporated to afford 10.85 g (93%) of crude **2** as a white powder containing only trace of ethylenediamine. ^{1}H-NMR ($CDCl_3$) δ 3.37 (br, 2H), 2.90 (t, 2H, *J*=5.9 Hz).

*2.2.2. (E)-5-(2-carbomethoxyvinyl)-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 $CHCl_3$/MeOH (9: 1, v/ v) showed the absence of starting material and formation of the product. The reaction mixture was filtered to remove the resulting precipitate palladium, and filtrate was evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$: MeOH= 9:1, v/v) to afford 5-carbomethoxyvinyl-2'-deoxyuridine as a white powder. ^{1}H –NMR (DMSO-d_6) δ 11.64 (bs, 1H, NH), 8.41 (s, 1H, H-6), 7.36 (d, 1H, vinyl, *J*= 15.8 Hz), 6.84 (d, 1H, vinyl, *J*= 15.8 Hz), 6.12 (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, OCH_3), 3.64-3.54 (m, 2H, H-5'), 2.17 (m, 2H, H-2').

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

4 (1.10 g, 3.54 mmol) was added to 3M NaOH (water/ ethanol= 1/ 1, 10 ml) and this mixture stirred at room temperature for 3h. HCl was powered into this solution on cooling ice bath to give white precipitate.

The precipitate was filtered washed with hexane, and sucked dry. The product was dried under vacuum to yield white powder. ^{1}H –NMR (DMSO-d_6) δ 11.61 (s, 1H, NH), 8.37 (s, 1H, H-6), 7.28 (d, 1H, vinyl, *J*= 15.8Hz), 6.12 (d, 1H, vinyl, *J*= 15.8 Hz), 6.12 (t, 1H, H-1', *J*=6.3 Hz), 5.20 (br, 1H, 3'-OH), 5.18 (br, 1H, 5'-OH, *J*=5.4 Hz), 4.25 (m, 1H, H-3'), 3.79 (m, 1H, H-4'), 3.59 (m, 2H, H-5'), 2.18 (m, 2H, H-2').

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

To a solution of **5** (0.79 g, 2.65 mmol) in anhydrous pyridine (12 ml) was added DMAP (0.03 g, 0.03 mmol) at ambient temperature. To a solution of DMTrCl (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. TLC 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 ($CHCl_3$: MeOH= 97:3, v/v) to afford **6** (0.68 g, 43 %) as white solid. ^{1}H-NMR ($CDCl_3$) 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, OCH_3), 3.40 (m, 2H, H-5'), 2.37-2.27 (m, 2H, H-2')

2.2.5.

*5'-O-(4,4'-dimethoxytrityl)-5-(E)-(N-[2-(2,2,2-trifluoro-acetylamino)-ethyl]-acrylamide)-2'-deoxyuridine (**7**)*

To a solution of **6** (0.30 g, 0.50 mmol) in dry CH_3CN (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 x 3) and water (30 ml), and washed with brine (20 ml x 2). The organic layer was collected, dried over anhydrous magnesium sulfate, and evaporated to dryness under reduce pressure. The crude was purified by column chromatography (ethyl acetate) to **7** as a white solid. ^{1}H NMR ($CDCl_3$) δ 8.13 (s, 1H, NH), 7.94 (s, 1H, 6-H), 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=6.6Hz, 1'-H), 5.55 (bs, 1H, 3'-OH), 4.49 (m, 1H, 3'-H), 4.07 (m, 1H, 4'-H), 3.75 (d, 6H, OCH_3), 3.47 (m, 2H, 5'-H), 3.33-3.30 (m, 4H), 2.27 (m, 2H, 2'-H); MALDI-TOF MS: calcd for $C_{37}H_{37}F_3N_4O_9Na[(M+Na)^+]$ 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-

*trifluoro-acetylamino)-ethyl]-acrylamide)-2'-deoxyuridine (**8**)*

To a solution of **7** (0.24 g, 0.32 mmol) in dry CH_3CN (5 ml) in a sealed bottle was added 0.45 M tetrazole in CH_3CN (0.71 ml, 0.32 mmol) and 2-cyanoethyl *N,N,N',N'*-tetra-isopropylphosphorodiamidite (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 $NaHCO_3$. 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 β̃(cyanoethyl)phosphoroamidite method on a controlled pore glass supports by using Applied Biosystems Model 3400 snthesizer. 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 1h ,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.

*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 μM) 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^+] 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 μM, strand conc.), ODN **3** (20 μM, strand conc.), ODN **4** (25 μM, 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 μWcm^{-2}) under otherwise identical conditions. After irradiation, 5 □L of aliquot was taken up and subjected to reverse phase HPLC analysis (elution with a solvent mixture of 50 mM ammonium formate, pH7.0, linear gradient over 15min from 6% to 9 % acetonitrile then over 15min from 9 % to 40 % acetonitrle 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 digesed 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 μM, strand conc.), ODN **6** (20 μM, strand conc.), ODN **4** (25 μM, 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 □L 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 digesed 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**. **7** was converted to the corresponding cyanoethyl phosphoramidite using a conventional method. The ^{CV}U with amino group-containing ODN (ODN **1**) was synthesized on an ABI3400 DNA synthesizer. Succinimidyl ester of Cy5 was coupled with ODN **1** to yield Cy5 and ^{CV}U-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 ^{CV}U-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** (Figure 1,2). 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 ^{CV}U side chain and the C5-C6 double bond of cytosine, giving rise to the formation of a cyclobutane structure as observed for ^{CV}U 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** (Figure 3,4). 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 (Figure 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 ^{CV}U-containing ODN. We determined the feasibility of photoligation of the Cy5 tethered ^{CV}U-containing ODN. We succeeded the synthesis of branched and Cy5-labeled ODN. These results indicate that method provides the site-specific and reversible labeling method of diagnostic sensing of nucleic acid sequences.

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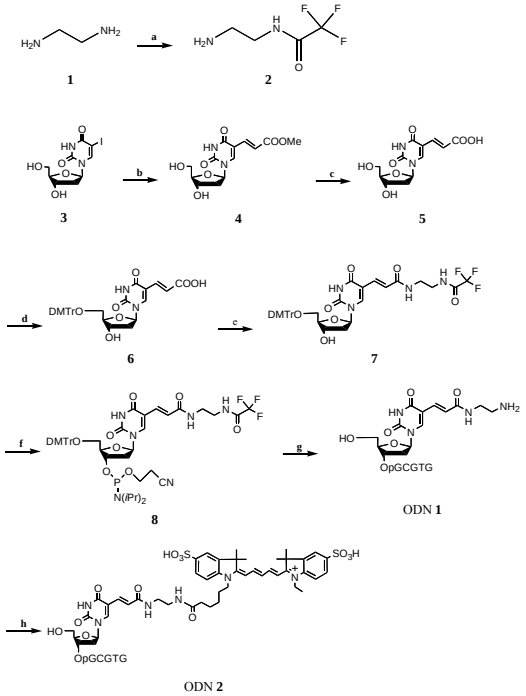
[19] The yield was calculated based on ODN **3**.

[20] The yield was calculated based on ODN **6**.

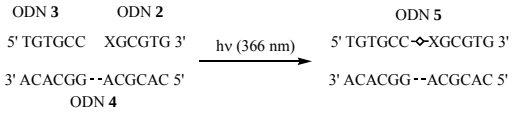
Figure Captions

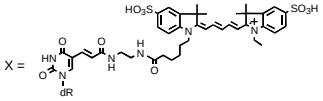
Scheme1



Reagents and Conditions: a; ethyl trifluoroacetate(1.0 eq.), -78 °C, b; methylacrylate (2.0 eq.), $Pd(OAc)_2$(0.05 eq.), PPh_3(0.15 eq.)/ dioxane, reflux 115 °C, 2h , c; 3M NaOH, r.t. 3 h, then 6M HCl, 0 °C, d; DMAP(0.1 eq.), DMTrCl (1.1 eq.)/ pyridine, 18 h, e; EDAC(1.2 eq.), NHS (1.2 eq.), **2**(1.0 eq.), f; $[(i\text{Pr})_2\text{N}]_2\text{POCH}_2\text{CH}_2\text{CN}$ (1.0 eq.), 1*H*-tetrazole(1.0 eq.)/ acetonitrile, g; DNA synthesizer, h; Cy5-NHS(1.0 eq.)/100 mM tetraborate buffer.

Scheme 2


ODN 3
ODN 2
ODN 5
5' TGTGCC XGCGTG 3'
hν (366 nm)
5' TGTGCC-◇-XGCGTG 3'
3' ACACGG--ACGCAC 5'
3' ACACGG--ACGCAC 5'
ODN 4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SO3H
N
N
HN
O
N
H
dR

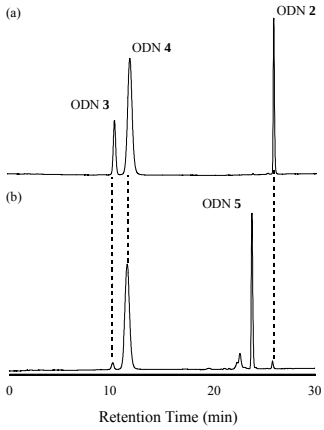


Figure 1. HPLC analysis of irradiated ODN **2** and ODN **3** in the presence of template ODN **4**; (a) before phototoirradiation, (b) after irradiation at 366 nm for 180 min at 0 °C (89 % yield)[19].

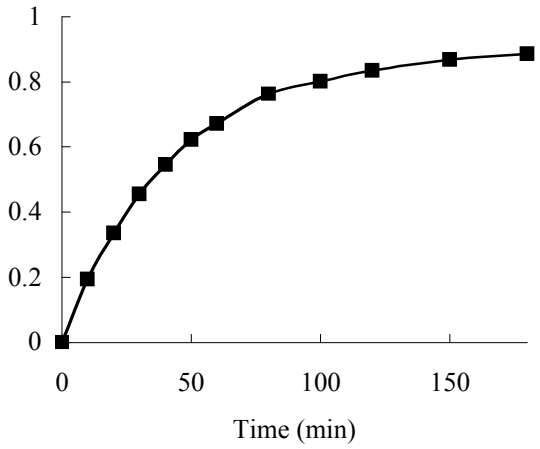
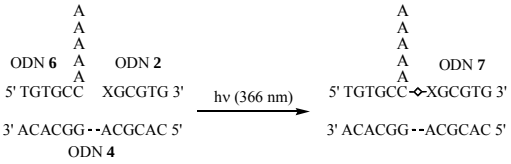


Figure 2. Photoligation rates of irradiated ODN **2** and ODN **3** in the presence of template ODN **4**

Scheme 3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A
ODN 6 A
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ODN 4
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--ACGCAC 5'
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--ACGCAC 5'
ODN 7

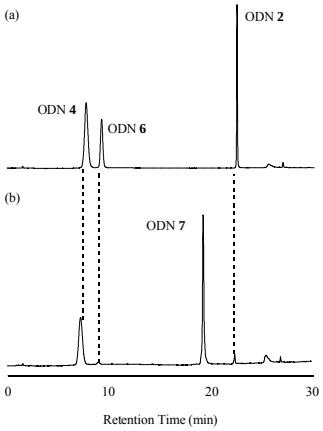

Figure 3. HPLC analysis of f irradiated ODN **2** and ODN **6** in the presence of template ODN **4**; (a) before phototoirradiation, (b) after irradiation at 366 nm for 180 min at 0 °C (90 % yield)[20].

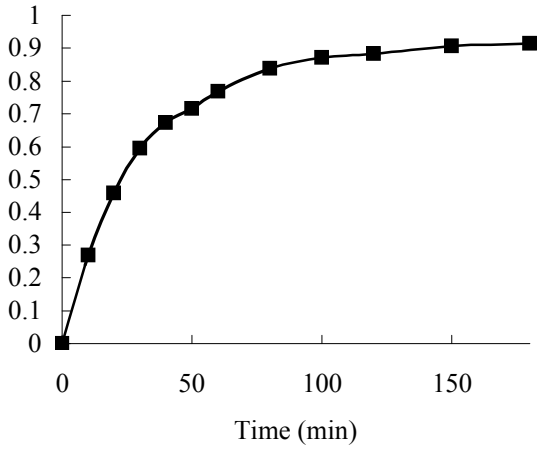

Figure 4. Photoligation rates of irradiated ODN **2** and ODN **6** in the presence of template ODN **4**

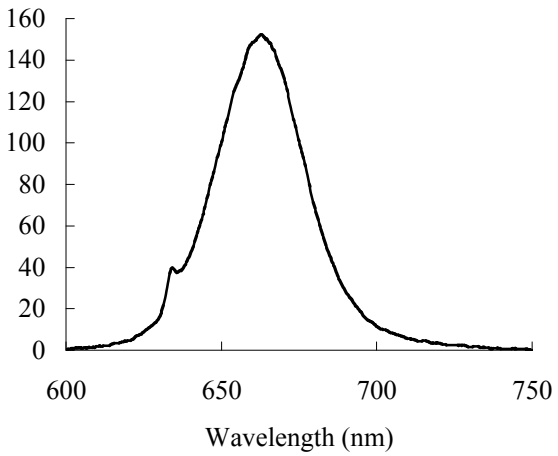

Figure 5. Fluorescent spectrum of ODN **7**. Excitation wavelength was 635 nm. λ_{em}= 663 nm.