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Click Chemistry to Construct Fluorescent Oligonucleotides for DNA Sequencing

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Received October 28, 2002

Abstract: "Click chemistry" 1,3-dipolar cycloaddition between alkynyl 6-carboxyfluorescein (FAM) and azido-labeled single-stranded (ss) DNA was carried out under aqueous conditions to produce FAM-labeled ssDNA in quantitative yield. The FAM-labeled ssDNA was successfully used as a primer to produce DNA sequencing products with single-base resolution in a capillary electrophoresis DNA sequencer with laser-induced fluorescence detection.

Synthetic oligonucleotides are the most important molecular tools for genomic research and biotechnology.[1] Modified oligonucleotides are widely used as primers for DNA sequencing[2] and polymerase chain reaction,[3] antisense agents for therapeutic applications,[4] molecular beacons for detecting genetic mutations,[5] and probes for measuring gene expression in DNA microarrays and gene chips.[6] The modification of either the 3′- and 5′-termini or an internal position of the oligonucleotides with a primary alkylamine group is a widely used method for introducing additional functional groups to the DNA.[7] Introduction of these functionalities into DNA can be achieved through the use of appropriate phosphoramidite reagents in solid-phase synthesis. Once a unique functional group is incorporated into the DNA, the functional group can be subsequently conjugated to the desired molecule by a selective chemical reaction. The succinimidyl ester of a fluorescent dye is widely used to couple with a primary amine group introduced into an oligonucleotide.[8] However, the coupling reaction requires aqueous conditions that can hydrolyze the succinimidyl ester moiety. To overcome this difficulty, phosphoramidite derivatives of fluorescent dyes were used to directly couple with the oligonucleotide in the solid-phase synthesis.[9] However, if the functional group is labile to the basic deprotection conditions used in the solid-phase DNA synthesis, the direct phosphoramidite approach cannot be used. Thus, there is still a need to develop coupling chemistry with high stability and high yield to modify DNA and other biomolecules. To this end, chemoselective modification of proteins and cell surfaces by the Staudinger ligation was developed.[10] Diels−Alder reaction was also explored for the selective immobilization of proteins.[11]

Ideal coupling functional groups (one on the DNA and the other on the molecule to be coupled) should be stable under aqueous reaction conditions. The coupling reaction should be highly chemoselective with a high yield, and the resulting linkage should be stable under biological conditions. Recently, Sharpless et al. defined "click chemistry" as a set of powerful, highly reliable, and selective reactions for the rapid synthesis of useful new compounds and combinatorial libraries through heteroatom links.[12] One of the click chemistry reactions involves coupling between azides and alkynes to form the triazole version of Huisgen's [2 + 3] cycloaddition family.[13] Mock et al.[14] discovered that cucurbituril could catalyze this 1,3-dipolar cycloaddition. This coupling chemistry was also used to form oligotriazoles and rotaxanes by Steinke et al.[15] The addition results in regioisomeric five-membered heterocycles.[16] This 1,3-dipolar cycloaddition chemistry is very chemoselective, only occurring between alkynyl and azido functional groups with high yield. In addition, the resulting 1,2,3-triazoles are stable at aqueous conditions and high temperature.

We recently explored the use of click chemistry 1,3-dipolar cycloaddition reaction to couple a fluorophore to DNA. We report here the synthesis of fluorescent single-stranded DNA (ssDNA) using the click chemistry and the application of the fluorescent ssDNA as a primer in the Sanger dideoxy chain termination reaction[17] to produce DNA sequencing fragments. We synthesized an oligonucleotide labeled by an azido group at the 5′-end as shown in Scheme 1. 5-Azidovaleric acid was synthesized

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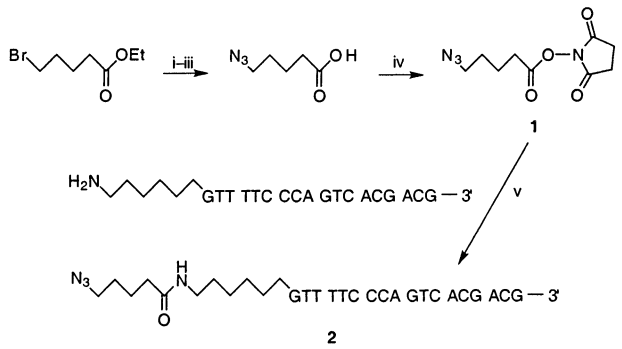
10.1021/jo026615r CCC: $25.00
Published on Web 12/21/2002

SCHEME 1[a]



[a] Reagents and conditions: (i) NaN_3, DMSO; (ii) NaOH, MeOH, H_2O; (iii) HCl; (iv) *N*-hydroxysuccinimide, EDC, CH_2Cl_2; (v) Na_2CO_3/$NaHCO_3$ buffer (pH 9.0), DMSO.

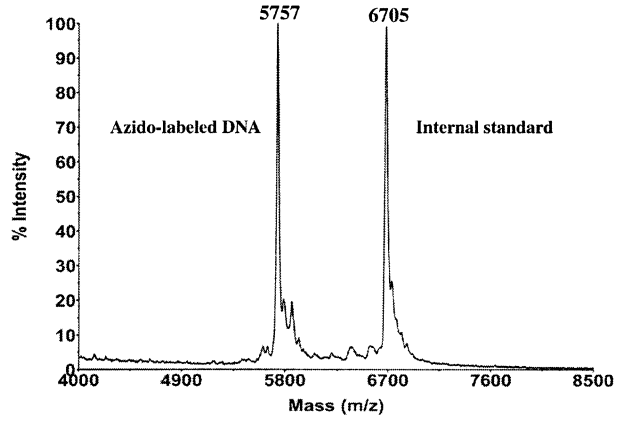


FIGURE 1. MALDI-TOF mass spectrum of **2**.

according to the literature[18] and activated as *N*-succinimidyl ester **1** (87%). The oligonucleotide 5′-amino-GTT TTC CCA GTC ACG ACG-3′ (M13-40 universal forward sequencing primer) was reacted with excess succinimidyl 5-azidovalerate **1** to produce the azido-labeled DNA **2**. After size-exclusion chromatography to remove excess starting material **1** and desalting with an oligonucleotide purification cartridge, the product was analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Figure 1 shows the MALDI-TOF MS spectrum of the isolated product, with a single major peak at 5757 Da that matched very well with the calculated value of 5758 Da for the azido-DNA **2**. This indicates that the starting material amino-DNA was quantitatively converted to the azido-DNA **2** (coupling yield ≈ 96%).

We then synthesized an alkynyl 6-carboxyfluorescein (FAM) **3** by reacting propargylamine with 6-carboxyfluorescein−NHS ester (Scheme 2). Click chemistry 1,3-dipolar cycloaddition between the alkynyl-FAM **3** and the azido-labeled DNA **2** was carried out at 80 °C in aqueous condition to produce FAM-labeled DNAs **4** and **5**. After the reaction, excess alkynyl-FAM **3** was removed by size-exclusion chromatography and the resulting FAM-labeled DNAs **4** and **5** were desalted with an oligonucleotide purification cartridge. We characterized products **4** and **5** by measuring their UV−vis absorption and MALDI-TOF MS spectra. Characteristic peaks with maxima of 500 nm (FAM) and 260 nm (DNA) were obtained by UV−vis measurement. The MALDI-TOF MS spectrum of **4** and **5** is shown in Figure 2. The mass peak of the azido-labeled DNA (5758 Da) almost completely disappeared, and a single major peak at 6170 Da corresponding to the cycloaddition reaction product (**4** and **5**, theoretical mass value of 6169 Da) was obtained with an isolated yield of 91%.

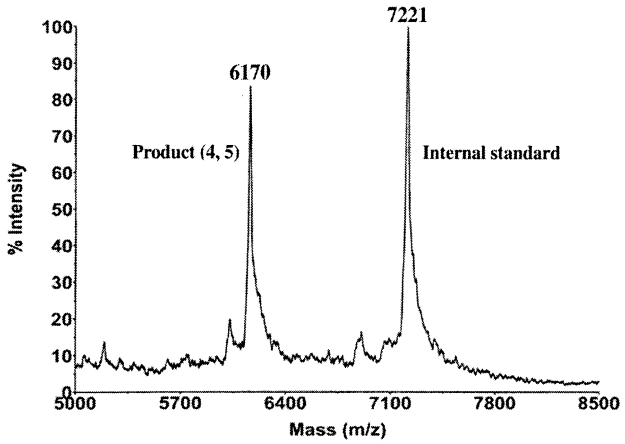
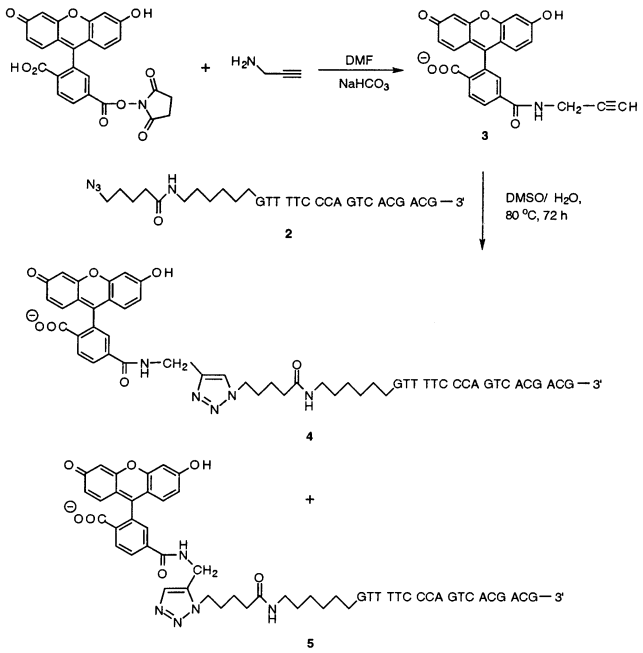


FIGURE 2. MALDI-TOF MS spectrum of **4** and **5**.

SCHEME 2



To demonstrate the utility of FAM-labeled oligonucleotides **4** and **5** constructed by the click chemistry for DNA analysis, we used them in the Sanger dideoxy chain termination method to produce DNA sequencing fragments terminated by biotinylated dideoxyadenine triphosphate (ddATP−Biotin) using PCR-amplified DNA as a template. Solid-phase capture using streptavidin-coated magnetic beads allows the isolation of pure DNA extension fragments free from false terminations.[19] These DNA fragments were analyzed by a capillary array electro-

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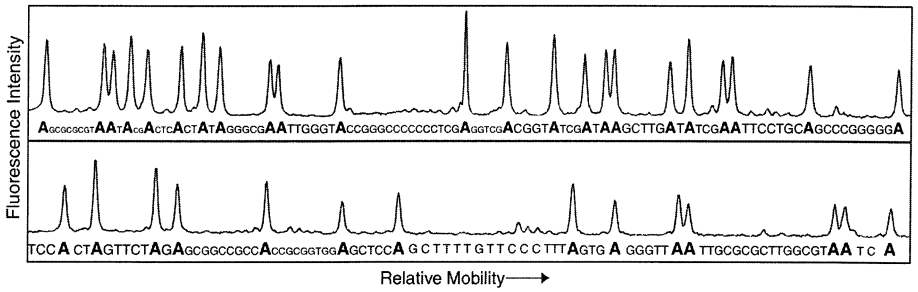

FIGURE 3. Electropherogram of DNA sequencing fragments generated with **4** and **5**.

phoresis (CAE) system[20] and resolved at single-base pair (bp) resolution to produce an electropherogram as shown in Figure 3. The peaks represent the FAM fluorescence emission from each DNA fragment that was extended from **4** and **5** and terminated by ddATP. This "A" sequencing ladder shown in Figure 3 matched exactly the sequence of the DNA template.

In summary, we have successfully demonstrated the use of the click chemistry to synthesize fluorescent oligonucleotides with high selectivity, high yield, and high stability. Without further purification by gel electrophoresis and HPLC that are required for conventional fluorescent oligonucleotide synthesis, the primer synthesized by the click chemistry can be used directly to produce DNA sequencing products with single-base resolution in a capillary electrophoresis DNA sequencer with laser-induced fluorescence detection. Further improvement of the methodology will allow reduced reaction time by attaching an electron-withdrawing functional group at the end of the triple bond.[12] It is expected that the optimized click chemistry will have potential applications in bioconjugation fields such as DNA covalent attachment on a chip, chemoselective protein modification, and immunoassays.

Experimental Section

Materials and General Procedures. The amino-C6-M13 (-40) forward primer (18mer) and internal mass standard oligonucleotides were commercially available and purified by HPLC. The ^{1}H and ^{13}C NMR spectra were recorded on 400 and 75 MHz NMR spectroscopic instruments, respectively. The high-resolution mass spectra (HRMS) were obtained under fast atom bombardment (FAB) conditions. UV−vis spectra of DNA samples were recorded in acetonitrile/water (1:1 volume ratio) at room temperature using quartz cells with path lengths of 1.0 cm.

Synthesis of Succinimidyl 5-Azidovalerate (1). 5-Azidovaleric acid was synthesized according to the published procedure.[18] 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC; 500 mg, 2.61 mmol) was added to a suspension of 358 mg (2.50 mmol) of 5-azidovaleric acid and 300 mg (2.61 mmol) of *N*-hydroxysuccinimide in CH_2Cl_2 (20 mL) at room temperature and stirred for 7 h, followed by the addition of H_2O. The separated CH_2Cl_2 phase was washed with H_2O and brine solution, dried over Na_2SO_4, and evaporated to yield 520 mg (87%) of succinimidyl 5-azidovalerate as a pale yellow liquid: IR (thin film) ν 2100, 1640 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.31 (t, 2H, J = 6.6 Hz), 2.81 (s, 4 H), 2.63 (t, 2H, J = 7.1 Hz), 1.86−1.68 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 169.1, 168.2, 50.8, 30.4, 27.8, 25.5, 21.8; HRMS (FAB$^+$) calcd for $C_9H_{13}O_4N_4$, 241.0937 (M + H$^+$); found, 241.0948.

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Synthesis of Azido-Labeled DNA (2). To incorporate the azido group at the 5′-end of the oligonucleotide, 10 nmol of amino-modified oligonucleotide in 40 μL of 0.25 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.0) was incubated for 12 h at room temperature with 10 μmol of succinimidyl 5-azidovalerate **1** in 12 μL of dimethyl sulfoxide. Unreacted succinimidyl 5-azidovalerate was removed by size-exclusion chromatography on a PD-10 column, and the resulting azido-labeled DNA was desalted with an oligonucleotide purification cartridge. The concentration of the collected azido-labeled DNA was measured by a UV−vis spectrophotometer, and the isolated yield was 96%.

Synthesis of 6-Carboxyfluorescein-propargylamide (Alkynyl FAM) (3). A solution of 3.4 μL (0.05 mmol) of propargylamine in DMF (0.5 mL) was added to a solution of 11 mg (0.023 mmol) of 6-carboxyfluorescein-NHS ester in DMF (0.5 mL) and 0.1 M $NaHCO_3$ solution (0.1 mL). After 5 h of stirring at room temperature, the solvent was removed under vacuum and the crude mixture was purified by a silica gel TLC plate (1:9 MeOH/$CHCl_3$) to give 8.0 mg (85%) of alkynyl FAM (R_f = 0.45) as a red oil: ^{1}H NMR (methanol-d_4) δ 8.01 (s, 2H), 7.60 (s, 1H), 6.94 (d, 2H, J = 9.1 Hz), 6.58−6.53 (m, 4H), 4.05 (d, 2H, J = 2.4 Hz), 2.50 (t, 1H, J = 2.2 Hz); ^{13}C NMR (methanol-d_4) δ 175. 3, 168.3, 158.5, 146.7, 136.9, 132.2, 129.9, 129.5, 128.7, 122.2, 121.0, 114.5, 104.0, 80.5, 72.2, 30.0; HRMS (FAB$^+$) calcd for $C_{24}H_{16}O_6N$, 414.0978 (M + 2H$^+$); found, 414.0997.

Synthesis of Fluorescent DNA by Click Chemistry (4 and 5). Azido-oligonucleotide (3.93 nmol) in 120 μL of water was reacted with a 150-fold excess of alkynyl FAM in 36 μL of DMSO at 80 °C for 72 h. Unreacted dye was removed by size-exclusion chromatography on a PD-10 column. The resulting fluorescent DNA was then desalted with an oligonucleotide purification cartridge, and the concentration was measured by a UV−vis spectrophotometer. The isolated yield of **4** and **5** was 91%.

Mass Spectrum of DNA. Mass measurement of oligonucleotides was performed using a MALDI-TOF mass spectrometer. DNA product (30 pmol) was mixed with 10 pmol of the internal mass standard, and the mixture was suspended in 2 μL of a 3-hydroxypicolinic acid matrix solution. A small amount (0.5 μL) of this mixture was spotted on a stainless steel sample plate, air-dried, and analyzed. The measurement was taken using a positive ion mode with a 25 kV accelerating voltage, a 94% grid voltage, and a 350 ns delay time.

PCR Amplification of Template. A PCR DNA product amplified from a pBluescript II SK(+) phagemid vector was used as a sequencing template because it has a binding site for the M13-40 universal primer. Amplification was carried out using the M13-40 universal forward and reverse primers in a 20 μL reaction, which contained 1X Accutaq LA reaction buffer, 250 pmol of each dNTP, 40 pmol of each primer, 0.5 unit of Jumpstart Red Accutaq LA DNA Polymerase, and 100 ng of the phagemid template. The reaction was performed in a DNA thermal cycler using an initial activation step of 96 °C for 1 min. This was followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min. At the end of the PCR reaction, 20 μL of an enzymatic mixture containing 5 units of shrimp alkaline phosphatase (SAP), 4 μL of 10X SAP buffer, 6 units of *E. coli* exonuclease I, and 10 μL of water were added to the PCR reaction to degrade the excess primers and dNTPs. The reaction

mixture was incubated at 37 °C for 90 min before the enzymes were heat inactivated at 72 °C for 30 min.

Generation and Detection of Sanger DNA Sequencing Fragments. A primer extension reaction was performed using the FAM-labeled primers **4** and **5** and the above PCR product. A 30 μL reaction mixture was made, consisting of 2.22 nmol of each dNTP, 37 pmol of Biotin-11-ddATP, 20 pmol of primer, 9 units of Thermo Sequenase DNA polymerase, 1X Thermo Sequenase reaction buffer, and 20 μL of PCR product. The reaction consisted of 30 cycles of 94 °C for 20 s, 50 °C for 20 s, and 60 °C for 90 s. Correctly terminated DNA fragments by Biotin-11-ddATP were purified from other reaction components using solid-phase capture according to the published method.[19] The fluorescent DNA fragments in 8 μL of formamide were electrokinetically injected at 3 kV into a capillary filled with linear polyacrylamide (LPA) gel in a capillary array fluorescent DNA sequencer and then separated at 8 kV in LPA buffer to produce a fluorescence electropherogram.

Acknowledgment. This research is supported by National Science Foundation (Sensing and Imaging Initiative Grant 0097793).

Supporting Information Available: ^{1}H and ^{13}C NMR spectral data for **1** and **3**, IR spectrum of **1**, and UV−vis spectrum of **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO026615R