Bioconjugate Chemistry

Article

pubs.acs.org/bc

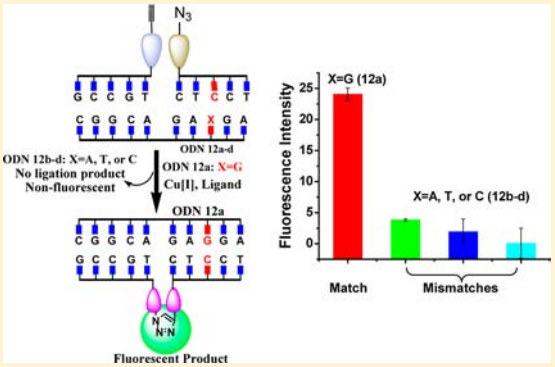
Template-Directed Fluorogenic Oligonucleotide Ligation Using "Click" Chemistry: Detection of Single Nucleotide Polymorphism in the Human p53 Tumor Suppressor Gene

Huabing Sun and Xiaohua Peng*

Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, 3210 North Cramer Street, Milwaukee, Wisconsin 53211, United States

Supporting Information

ABSTRACT: A novel nonfluorescent alkyne-modified coumarin phosphoramidite was synthesized and successfully incorporated into oligonucleotides, which were then used in highly efficient DNA interstrand cross-linking and ligation reactions via "click" chemistry. The template-directed fluorogenic ligation "click" chemistry reaction was used for single nucleotide polymorphism analysis, where the target DNA catalyzes the ligation of two nonfluorescent probes to generate a fluorescent product. The upstream oligonucleotide probe is a nonfluorescent alkyne-modified coumarin and the downstream probe is an azide-modified oligonucleotide. When bound to a fully complementary template, the oligonucleotides ligated to produce a fluorescent product with a fluorophore at the ligation point. Wild-type and mutant p53 alleles were used to demonstrate that template-directed fluorogenic oligonucleotide ligation is sequence-specific and is capable of single nucleotide discrimination under mild conditions, even without the removal of unreacted probes.



INTRODUCTION

The sequence-specificity and high efficiency of DNA-directed chemical reactions have enabled their use in a wide variety of applications.[1,2] Interstrand cross-linking (ICL) and ligation are important components of DNA-templated reactions. Induction of ICL by chemical agents or photoactivation disrupts cell homeostasis and replication, and is therefore an important strategy in cancer therapy.[3] In addition, cross-linked DNA duplexes have been used to study DNA repair,[4] gene regulation,[5] and the reversible control of DNA hybridization,[6] and have also been utilized as aptamers and decoys to sequester DNA-binding proteins.[7] On the other hand, DNA-templated ligation reactions have been used to monitor and detect specific nucleic acids[2,8−11] or proteins. [12] Several important issues, such as bioorthogonality, functional group tolerance, and detection of the formed product, must be considered when designing DNA-diagnostic chemical reactions. The "click" chemistry reaction is a regioselective and bioorthogonal process that is clean, fast, and high-yielding; can be operated in aqueous solutions; is tolerant of other functional groups; and requires minimal workup and purification.[13,14] Therefore, this method is ideal for addressing the issues related to DNA-diagnostic chemical reactions.

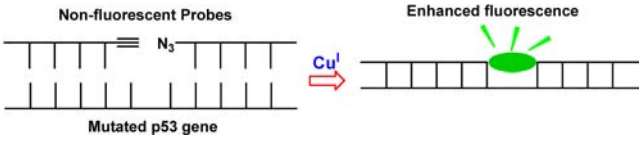
The Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) reaction, which is the most prominent example of click chemistry,[13] has attracted particular attention for use in DNA cross-linking and ligation, as well as a variety of other biological reactions.[14] "Click" chemistry has been used to cross-link complementary DNA strands through modified nucleobases or sugar moieties.[15−22] CuAAC reactions have also been used for peptide-nucleic acid ligations by our group and others.[23,24] Recently, fluorogenic CuAAC reactions between nonfluorescent alkynes and azides that form triazole complexes with enhanced fluorescence have been optimized. These reactions generate a fluorescent signal that differs between the starting material and the resulting product[25−28] and have been widely used in bioconjugation labeling[29−31] and fluorogenic probing.[32−34] However, to our knowledge, the fluorogenic "click" method has not yet been used in DNA-directed reactions and no applications in this field have been reported.

In this study, we used the CuAAC reaction to develop a template-dependent fluorogenic "click" reaction capable of single nucleotide discrimination and demonstrated its ability to detect specific DNA sequences. DNAs containing nonfluorescent alkyne-modified coumarin or an azide group were employed as probes for DNA-templated ligation and ICL, and were used to detect single nucleotide polymorphisms (SNPs) in the human p53 tumor suppressor gene. The fluorogenic "click" reaction occurred only in the presence of the mutant p53 gene (Scheme 1).

Received: April 2, 2013
Revised: May 24, 2013
Published: June 11, 2013

 © 2013 American Chemical Society dx.doi.org/10.1021/bc4001678 | *Bioconjugate Chem.* 2013, 24, 1226−1234

Scheme 1. Detection of a Mutated p53 Gene by a Fluorogenic "Click" Reaction



EXPERIMENTAL PROCEDURES

General Methods and Materials. Reagents were obtained from Aldrich or Fisher Scientific and used as received without further purification. Columns and phosphoramidites used for the synthesis of oligodeoxynucleotides (ODNs) were purchased from Glen Research. T4 polynucleotide kinase was purchased from New England Biolabs. [γ-^{32}P]-ATP was obtained from Perkin-Elmer Life Sciences. Radiolabeling was carried out according to the standard protocols.[35] Quantification of radiolabeled ODNs was carried out using a Molecular Dynamics Phosphorimager equipped with ImageQuant version 5.2 software. Fluorescence spectra were recorded on a Perkin-Elmer LS55 Fluorescence Spectrometer using the quartz cell with 10 mm path lengths at room temperature. The concentrations of ODNs in water were determined by Varian CARY-100 BIO UV–vis spectrophotometer at 260 nm. 7-Ethynyl-4-hydroxymethyl-chromen-2-one (**6**) and succinimidyl 5-azidovalerate (**10**) were prepared as described before.[23,36,37] Detailed protocols for synthesizing **6** and **10** are described in the Supporting Information. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on a Bruker DRX 300 MHz spectrophotometer. Chemical shifts (δ) are reported in ppm relative to Me_4Si (^{1}H and ^{13}C) or H_3PO_4 (^{31}P). Coupling constants (J) are reported in Hz. Mass spectrometry was performed in Prof. Jack W. Szostak's lab at the Harvard University and University of California-Riverside Mass Spectrometry Lab.

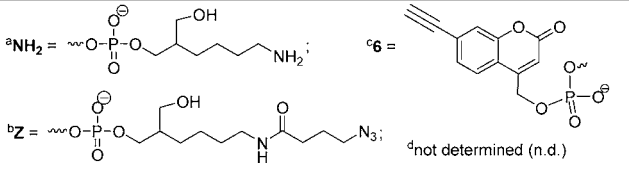
7-Ethynyl-4-hydroxymethylchromen-2-*O*-(2-cyanoethyl-*N*,*N*-diisosopropyl) Phosphoramidite (7). To a solution of **6** (20 mg, 0.1 mmol) in dichloromethane (2 mL), *N*,*N*-diisopropylethylamine (DIPEA) (33.6 μL, 0.18 mmol), and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (33.6 μL, 0.15 mmol) were added under an atmosphere of argon. The reaction mixture was stirred at room temperature for 3 h and diluted with EtOAc (30 mL). The organic layer was washed with water (20 mL × 2) and saturated aqueous NaCl (20 mL), and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The crude product was purified by column chromatography (EtOAc:hexane:Et_3N = 66:33:1) yielding **7** as a white solid (28 mg, 70%). ^{31}P ($CDCl_3$, 300 MHz): δ 149.38. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.50 (d, J = 8.1 Hz, 1 H), 7.46 (s, 1 H), 7.28 (d, J = 8.1 Hz, 1 H), 6.61 (s, 1 H), 4.87 (t, J = 6.3 Hz, 1 H), 3.66–3.99 (m, 4 H), 3.28 (s, 1 H), 2.67 (t, J = 6.3 Hz, 1 H), 1.25 (d, 12 H). ^{13}C NMR ($CDCl_3$): δ 160.4, 153.2, 151.7, 151.6, 127.9, 125.6, 120.6, 117.8, 117.6, 113.3, 82.0, 61.6, 61.3, 58.5, 58.2, 43.6, 43.4, 24.7, 20.6, 20.5.

Preparation of Oligodeoxynucleotides. ODNs were synthesized via the standard phosphoramidite approach using an Applied Biosystems model 394 instrument on a 1.0 μmol scale. Unless otherwise specified, deprotection of the nucleobases and phosphate moieties as well as cleavage of the linker were performed under mild deprotection conditions (40% aq. $MeNH_2$ and 28% aq. NH_3, v/v = 1:1) at room temperature for 2 h. The crude ODNs were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE). Compound **7** was incorporated into ODNs using the phenoxyacetyl (PAC) phosphoramidites. Deprotection and cleavage were carried out with 28% aq. NH_3 at room temperature for 2 h. C7-Aminoalkyl ODNs were prepared using 3′-amino-modifier C7 CPG columns. Deprotection and cleavage of the ODNs were carried out with 28% aq. NH_3 at 55 °C for 12 h. To introduce the azido group at the 3′-terminus, 100 nmol of C7-aminoalkyl ODNs in 0.5 M Na_2CO_3/$NaHCO_3$ buffer (pH 8.75, 40 μL) were incubated at room temperature for 4 h in the presence of succinimidyl-4-azidobutyrate (**10**) (20 μmol) in DMSO (12 μL) (Scheme 3).[22]

Determination of DNA Melting Temperature. The thermal stability of DNA duplexes was determined on a Varian CARY-100 BIO UV–vis spectrophotometer with the Cary Win UV Thermal program using a 1.0 cm path-length cell. All measurements were performed in 10 mM potassium phosphate buffer (pH 7), 100 μM ethylenediaminetetraacetic acid

Table 1. Oligodeoxynucleotides Used in the Study

Entry	ODN sequence	Mass (Calcd.)	Mass (Found)[d]
ODN **1**	3′-dCGGAGGCCAAGT		n.d.
ODN **2**	3′-dACTTGGCCTCCG		n.d.
[a]ODN **3**	3′-d**NH$_2$**ACTTGGCCTCCG		n.d.
[b]ODN **4**	3′-d**Z**ACTTGGCCTCCG	3917.5	3916.9
[c]ODN **5**	3′-dCGGAGGCCAAGT**6**	3957.5	3957.4
[c]ODN **6**	3′-dCGGAGACCAAGT**6**	3941.5	3941.6
[a]ODN **7**	3′-d**NH$_2$**CTTGGCCT		n.d.
[b]ODN **8**	3′-d**Z**CTTGGCCT	2696.7	2696.6
[a]ODN **9**	3′-d**NH$_2$**CTTGGCCTC		n.d.
[b]ODN **10**	3′-d**Z**CTTGGCCTC	2985.9	2985.9
[c]ODN **11**	3′-dCGTACCCGCCGT**6**	3844.4	3844.0
ODN **12a-d**	3′-dTACCCGGAG**X**CCAAG TACGGCGGGTACGTCCT **12a**: **X** = G; **12b**: **X** = A; **12c**: **X** = T; **12d**: **X** = C		n.d.
ODN **13**	3′-dAGGCCAAGT		n.d.
ODN **14a-d**	3′-dGAG**X**CCAAGT **14a**: **X** = G; **14b**: **X** = A, **14c**: **X** =T; **14d**: **X** =C		n.d.



dx.doi.org/10.1021/bc4001678 | Bioconjugate Chem. 2013, 24, 1226–1234

(EDTA), and 100 mM NaCl, with 4 μM + 4 μM single strand concentration. The concentrations of the ODNs were determined by measuring the absorbance at 260 nm. The extinction coefficients at 260 nm of the ODNs were calculated from the sum of the extinction coefficients of the monomeric 2′-deoxyribonucleotides. Samples were heated at 1 °C min^{-1} from 4 to 80 °C and the absorbance at 260 nm was measured at 1.0 °C steps. Three independent samples have been determined to get the melting temperatures of DNA duplexes.

PAGE Analysis of Template-Directed "Click" Ligation Reaction and the Kinetics Study. The 5′-^{32}P-labeled ODN **8** (ODN **10** or ODN **4**) (500 nM), ODN **11** (750 nM), and templates (ODN **12a**−**d**, 750 nM) were hybridized in 100 mM NaCl and 10 mM potassium phosphate (pH 7). The "click" ligation of DNA duplexes (50 nM) was performed in the same buffer (10 mM pH 7 potassium phosphate, 100 mM NaCl) under the optimized conditions (80 mM THPTA/4 mM $CuSO_4$/40 mM Na-ascorbate) at the designed temperature. A control reaction was carried out in the absence of templates. Aliquots were taken at the prescribed times and immediately quenched with the equal volume of 95% formamide loading buffer, and stored at −20 °C until subjecting to 20% denaturing PAGE analysis. For kinetics study, three independent samples were studied with the same procedures mentioned above.

Isolation of the Interstrand Cross-Link Product. ODN **4** (20 μM) and its complementary strand ODN **5** (1.5 equiv) were hybridized in 100 mM NaCl and 10 mM potassium phosphate buffer (pH 7). The cross-linking reaction of DNA duplex (10 μM, 200 μL) was carried out in 10 mM potassium phosphate (pH 7) and 100 mM NaCl in the presence of 80 mM THPTA/4 mM $CuSO_4$/40 mM Na-ascorbate at room temperature for 1 h. Then, 2 M NaCl solution (200 μL) and cold ethanol (1.2 mL) were added, the mixture was incubated at −80 °C for 30 min and DNA was precipitated by centrifugation at 15000 *g* for 6 min at room temperature. ODN **15** was purified by 20% denaturing PAGE. The band containing the cross-linked product was cut, crushed, and eluted with 200 mM NaCl, 20 mM EDTA (2.0 mL). The crude product was further purified by C18 column eluting with H_2O (3 × 1.0 mL) followed by MeOH:H_2O (3:2, 1.0 mL). ODN **15** was characterized by MALDI-TOF and fluorescence spectroscopy. Its fluorescence (10 μM) was measured in water on a Perkin-Elmer LS55 Fluorescence Spectrometer.

Detection of Interstrand Cross-Linking by Fluorescence Spectroscopy. The "click" reaction of DNA duplexes (0.2 μM, 200 μL) was performed in 10 mM potassium phosphate (pH 7) and 100 mM NaCl in the presence of 80 mM THPTA/4 mM $CuSO_4$/40 mM Na-ascorbate for 1 h. The reaction mixture was precipitated by adding 2.0 M NaCl (30 μL) and cold ethanol (690 μL), keeping at −80 °C for 30 min. The DNA precipitates were collected by centrifugation at 15000 *g* for 6 min at room temperature. The precipitation procedures were repeated twice. Finally, the DNA precipitates were dissolved in 50 μL H_2O and subjected to gel-filtration on an Illustra G-25 column (GE Healthcare) to remove the catalyst and salts. The final product was dissolved in 100 μL water for fluorescence measurement.

Isolation of "Click" Ligation Products. ODN **10** (20 μM), ODN **11** (1.5 equiv), and ODN **12a** (1.5 equiv) were hybridized in 100 mM NaCl and 10 mM potassium phosphate (pH 7). The "click" ligation of DNA duplexes (10 μM, 170 μL) were carried out in 10 mM potassium phosphate (pH 7) and 100 mM NaCl under the optimized conditions (80 mM THPTA/4 mM $CuSO_4$/40 mM Na-ascorbate, room temperature, 3 h). The reaction mixture was precipitated by adding 2.0 M NaCl (30 μL), cold ethanol (600 μL), and keeping at −80 °C for 30 min. The precipitates were dissolved in 50 μL H_2O and 50 μL loading buffer (10 mM EDTA, 95% formamide). The ligation products were purified by 20% denaturing PAGE. The band containing ligated product was cut, crushed, and eluted with 200 mM NaCl, 20 mM EDTA (2.0 mL). The crude product was further purified by C18 column eluting with H_2O (3 × 1.0 mL) followed by MeOH:H_2O (3:2, 1.0 mL). The ligation product was characterized by MALDI-TOF. The fluorescence of ODN **16** (10 μM) was measured in water on a Perkin-Elmer LS55 Fluorescence Spectrometer.

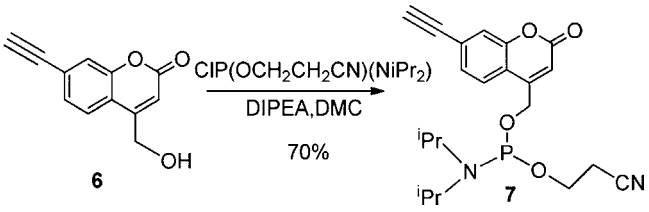
Detection of SNPs in P53 Tumor Suppressor Gene with DNA-Templated Fluorogenic "Click" Reaction. ODN **10** (0.4 μM) and ODN **11** (1.5 equiv) were hybridized with DNA templates (ODNs **12a**−**d**, 1.5 equiv) in 100 mM NaCl and 10 mM potassium phosphate (pH 7). The "click" ligation reaction of DNA duplexes (0.2 μM, 200 μL) was carried out in 10 mM potassium phosphate (pH 7) and 100 mM NaCl under the optimized conditions (80 mM THPTA/4 mM $CuSO_4$/40 mM Na-ascorbate, room temperature, 3 h). After 3× precipitations (30 μL 2 M NaCl/690 μL ethanol, −80 °C, 30 min), the DNA precipitates were dissolved in 50 μL water and subjected to gel-filtration on an Illustra G-25 column (GE Healthcare). The DNA was reprecipitated, dissolved in 100 μL water, and the fluorescence determined.

RESULTS AND DISCUSSION

Experimental Design and Synthesis of Oligodeoxynucleotides. The aim of this study was to develop a simple and effective DNA-templated fluorogenic reaction using "click" chemistry, to demonstrate its ability to cross-link and ligate DNA, and to evaluate its application for the detection of specific nucleic acid sequences. Nonfluorescent DNA probes that could be covalently bound via a CuAAC reaction were designed. The use of nonfluorescent probes ensures a low background signal that improves the sensitivity of DNA detection. Covalent attachment between the two probes produces a permanent fluorescent signal and does not require removal of unreacted probes by stringent washing. Initially, we chose coumarin derivatives as fluorogenic reporters because they are biocompatible and easy to prepare, and their fluorescence intensities can be tuned by varying the electronic properties of the substituents at the aromatic rings.[26]

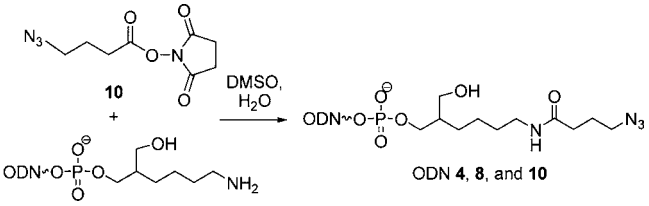
Coumarin analogues containing an alkyne group at the 7-position or an azido group at the 3-position are nonfluorescent but can undergo a "click" reaction to generate a fluorescent product via formation of a triazole moiety.[26] Azide groups interact with phosphoramidite substrates via the Staudinger reaction and are not compatible with solid-phase DNA synthesis;[38] therefore, alkyne-modified coumarin was used as the reporter, which was incorporated at the 5′-terminus of DNA probes. The phosphoramidite building block (Compound **7**) of 7-ethynyl-4-hydroxymethylchromen-2-one (Compound **6**) was prepared as shown in Scheme 2. Compound **6** was synthesized as described previously (see Supporting Information).[36] PAC phosphoramidites were employed for synthesizing oligodeoxynucleotides (ODNs) containing alkyne-modified coumarin because a very mild deprotection condition can be used, which prevents the decomposition of the functionalized DNA probes.

Scheme 2. Synthesis of the Alkyne-Modified Coumarin Phosphoramidite Building Block 7 from 7-Ethynyl-4-hydroxymethylchromen-2-one (6)



As shown in Scheme 3, ODNs containing an azide group at the 3′-terminus (ODNs **4**, **8**, and **10**) were generated as a result

Scheme 3. Synthesis of ODNs Containing an Azide Group



of the reaction between 3′-amino-modifier C7-ODNs and succinimidyl-4-azidobutyrate (Compound **10**). Compound **10** was synthesized from methyl 4-bromobutanoate (see Supporting Information). All ODNs were purified by 20% denaturing PAGE. The generation of ODNs containing alkyne-modified coumarin or an azide group was confirmed by mass spectrometry (Figures S2–6, Supporting Information).

Effect of the Alkyne-Modified Coumarin and Azide Groups on ODN Duplex Stability. The efficiency and sequence-selectivity of DNA-templated chemical reactions depend on the formation of a stable DNA duplex and the mismatch discrimination of the modified DNA probes, respectively. In order for coumarin or an azide group to be useful components of a probe capable of specific detection of a complementary sequence, they must not disrupt the mismatch discrimination ability of the probe. Therefore, the duplex stabilities of ODNs containing an alkyne-modified coumarin moiety or an azide group were examined (Table 2). The melting temperature (T_m) of the duplex formed between ODNs lacking coumarin moieties or azide groups was 53.1 °C (ODN **2**-ODN **1**). Addition of a coumarin moiety increased the T_m by 3.6 °C (ODN **2**-ODN **5**), while addition of an azide

Table 2. Duplex Melting Temperatures[a]

Duplex	T_m (°C)	ΔT_m (°C)
5′-d(GCC TC C GGT TCA) (ODN **2**) 3′-d(CGG AGG CCAGT) (ODN **1**)	53.1 ± 0.3	
5′-d(GCC TC C GGT TCA**Z**) (ODN **4**) 3′-d(CGG AGG CCA AGT) (ODN **1**)	52.7 ± 0.4	−0.4
5′-d(GCC TC C GGT TCA) (ODN **2**) 3′-d(CGG AGG CCA AGT**6**) (ODN **5**)	56.7 ± 0.4	+3.6
5′-d(GCC TC C GGT TCA**Z**) (ODN **4**) 3′-d(CGG AGG CCA AGT**6**) (ODN **5**)	57.7 ± 0.8	+4.6
5′-d(GCC TC C GGT TCA**Z**) (ODN **4**) 3′-d(CGG AG**A** CCA AGT**6**) (ODN **6**)	40.3 ± 0.3	−17.4

[a]Measured in a buffer containing 0.1 M NaCl, 10 mM potassium phosphate (pH 7), and 4 μM of each single-stranded ODN.

group (ODN **4**-ODN **1**) slightly decreased the duplex stability (ΔT_m = −0.4 °C, relative to the ODN **2**-ODN **1** duplex). The ODN **4**-ODN **5** duplex, which contained a coumarin moiety and an opposing azide group, showed the greatest stability (ΔT_m = 4.6 °C). The addition of the coumarin moiety and the azide group to the ODN probes did not affect the mismatch discrimination between dC and dA (ΔT_m = −17.4; ODN **4**-ODN **6**). These data suggest that both azide and coumarin moieties can be used as reporters for ODN probes designed to detect SNPs.

Fluorogenic DNA Interstrand Cross-Linking via "Click" Reaction. The ODN probes described above were designed to target the region surrounding codon 248 in exon 7 of the p53 gene. This gene is often mutated in human cancer and an A to G nucleotide transition is frequently observed at codon 248.[39,40] ODN **4** was designed to discriminate between a perfectly matched sequence containing dG at codon 248 (ODN **5**) and a mismatched sequence containing dA at the same position (ODN **6**). A fluorogenic DNA ICL reaction using "click" chemistry was performed using ODN **4**, which contained an azide group at the 3′-terminus, and ODN **5**, which contained an alkyne-modified coumarin moiety at the 5′-terminus; the resulting duplex was named ODN **15** (Figure 1A). To ensure complete formation of the duplex, the "click"

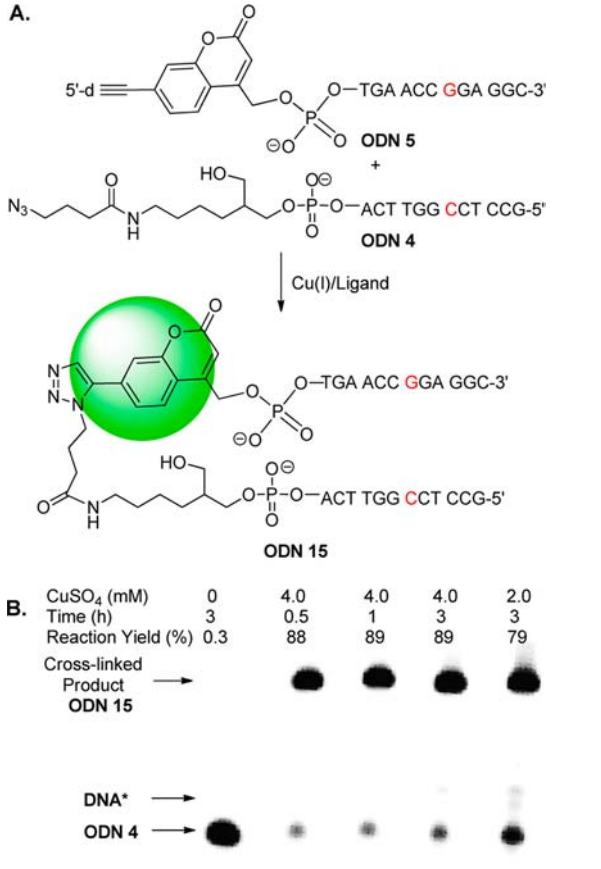


Figure 1. (A) Fluorogenic DNA ICL reaction between ODN **4** and ODN **5**. The mutated nucleotide in codon 248 of the p53 sequence is shown in red. (B) Phosphorimage autoradiogram of denaturing PAGE analysis of the "click" reaction between ODN **4** and ODN **5** ([ODN **4**] = 50 nM, [ODN **5**] = 75 nM) (ODN **4** was labeled with ^{32}P at the 5′-terminus). The reaction was performed at room temperature with THPTA, $CuSO_4$, and sodium ascorbate at a ratio of 20:1:10.

dx.doi.org/10.1021/bc4001678 | Bioconjugate Chem. 2013, 24, 1226–1234

reaction was performed in 10 mM potassium phosphate buffer (pH 7) containing 100 mM NaCl. Gel electrophoresis was initially used to examine the efficiency of the ICL between ODNs **4** and **5**. ODN **4** was labeled at the 5′-terminus with ^{32}P and the cross-linking reaction was performed at room temperature in the presence of Cu[I] and Tris-(hydroxypropyltriazolylmethyl)amine (THPTA). Cu[I] was generated in situ from $CuSO_4$ and sodium ascorbate, and was bound to its water-soluble ligand THPTA, which greatly reduced the degradation of ODNs and increased the yield of the "click" reaction.[41,42] The DNA ICL reaction between ODN **4** and ODN **5** was highly efficient; a yield of approximately 90% was produced within 30 min (Figure 1B, lane 2). A control reaction performed in the absence of $CuSO_4$, sodium ascorbate, and THPTA did not produce a cross-linked product. Reduction of the catalyst concentration produced a lower yield (79%; Figure 1B, lane 5). The rate of DNA ICL followed first-order kinetics (k_{ICL} = (1.1 ± 0.2) × 10^{-3} s^{-1}; $t_{1/2}$ = 10.5 min; Figure S7B, Supporting Information). Apart from formation of the cross-linked product, a byproduct (DNA*) was observed (Figure 1B, lanes 2−5), which is likely caused by the side reaction between azide-modified DNA and the ligand as it was not formed in the absence of the ligand (Figure 1B, lane 1). This has been reported in our previous work.[23] The cross-linked product (ODN **15**) was purified by gel electrophoresis and characterized by MALDI-TOF (m/z = 7914.1 $[M+K]^+$; calcd. m/z = 7914.0; Figure S8, Supporting Information).

Fluorescence spectroscopy revealed that the fluorescence intensity of the cross-linking product ODN **15** was five times higher than that of a mixture of unreacted ODN **4** and ODN **5** (10 μM each) (Figure 2A). This result is consistent with a previous study, which demonstrated that addition of an alkyne group at the 7-position quenches the fluorescence of a coumarin moiety, while the formation of a triazole moiety restores the fluorescent signal.[36] Our results indicated that the restored fluorescence was not quenched by duplex DNA; therefore, alkyne-modified coumarin is a suitable reporter group for DNA-templated fluorogenic reactions. Fluorescence spectroscopy was also used to monitor the DNA ICL reaction. The fluorescence of the reaction mixture was measured before and after completion of the "click" reaction. Enhanced fluorescence intensity was observed upon addition of the catalyst (80 mM THPTA, 4 mM $CuSO_4$, and 40 mM sodium ascorbate) (Figure 2B), demonstrating that the DNA cross-linking reaction can be monitored by fluorescence spectroscopy.

Finally, the selectivity of the DNA-templated fluorogenic "click" reaction, which depended on the reaction temperature and duplex stability, was examined. A cross-linked product containing a single nucleotide mismatch (Figure S9B, Supporting Information) was formed after incubation of ODN **4** with ODN **6** at room temperature. The cross-linking yield was comparable to that obtained with ODN **4** and its perfectly matched ODN **5** complement. However, the discriminative ability of ODN **4** toward ODN **5** and ODN **6** (approximately 10:1) was achieved when the reaction was performed at 45 °C, which is slightly higher than the T_m of the mismatched duplex (Figure 3). The perfectly matched duplex (ODN **4**-ODN **5**) resulted in efficient ICL (yield of 83%), while the ICL yield of the mismatched duplex (ODN **4**-ODN **6**) was less than 8%. These data indicate that the DNA-templated fluorogenic "click" reaction is sequence-specific and has the potential to be used for SNP detection.

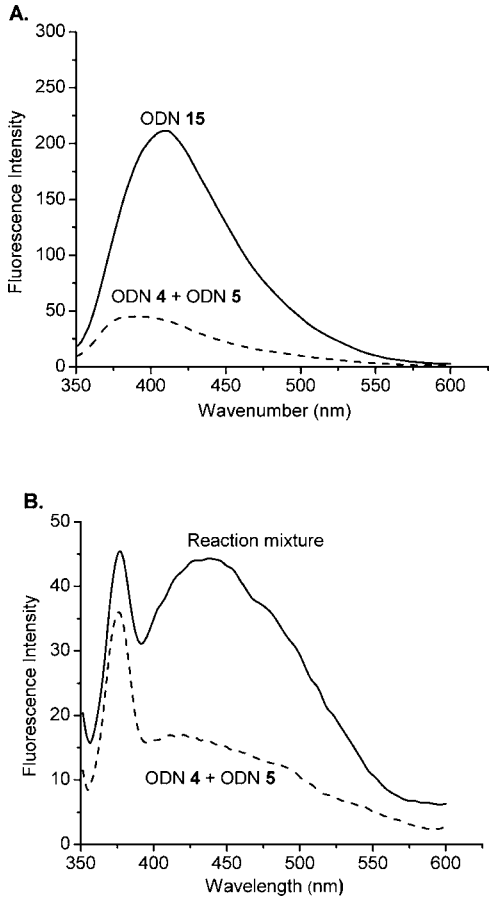


Figure 2. (A) Fluorescence emission spectra of the gel-purified cross-linking product (ODN **15**; 10 μM) (solid line) and a mixture of unreacted ODN **4** (10 μM) and ODN **5** (10 μM) (dashed line) (λ_{ex} = 330 nm, slit width = 10 nm; λ_{em} = 410 nm, slit width = 10 nm). (B) Fluorescence emission spectra of a reaction mixture containing ODN **4** (0.4 μM) and ODN **5** (0.4 μM) in the presence (solid line) or absence (dashed line) of 80 mM THPTA, 4 mM $CuSO_4$, and 40 mM sodium ascorbate (λ_{ex} = 330 nm, slit width = 5 nm; λ_{em} = 410 nm, slit width = 15 nm). Reactions were incubated for 1 h at room temperature; the catalyst, ligand, and salts were removed by precipitation and by passing the reaction mixture through an Illustra G-25 column. The spectra were measured in water (pH 7) at room temperature.

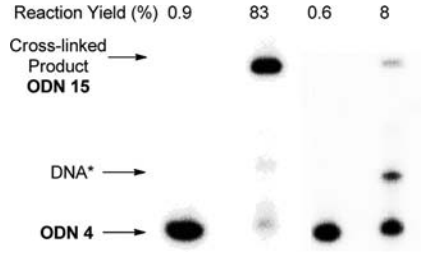


Figure 3. Phosphorimage autoradiogram of denaturing PAGE analysis of the "click" cross-linking reaction capable of single nucleotide discrimination. Lane 1, ODN **4**-ODN **5** in the absence of THPTA, $CuSO_4$, and sodium ascorbate; lane 2, ODN **4**-ODN **5** in the presence of THPTA, $CuSO_4$, and sodium ascorbate; lane 3, ODN **4**-ODN **6** in the absence of THPTA, $CuSO_4$, and sodium ascorbate; lane 4, ODN **4**-ODN **6** in the presence of THPTA, $CuSO_4$, and sodium ascorbate. The "click" reactions were performed at 45 °C for 30 min; [ODN **4**] = 50 nM, [ODN **5**] = 75 nM, [ODN **6**] = 75 nM (ODN **4** was labeled with ^{32}P at the 5′-terminus).

dx.doi.org/10.1021/bc4001678 | Bioconjugate Chem. 2013, 24, 1226−1234

DNA-Templated Fluorogenic "Click" Ligation and Single Nucleotide Discrimination. Our preliminary experiments established that the fluorogenic "click" ICL reaction can be used to detect SNPs in DNA sequences. However, DNA cross-linking has some limitations because it requires both probe and target to be modified with either an azide or an alkyne moiety, and modification of the target ODNs at specific positions is difficult. This problem can be overcome by developing a template-directed fluorogenic "click" ligation reaction, in which two modified probes that are complementary to a native target are employed. Development of DNA probes that are capable of undergoing sequence-specific fluorogenic "click" ligation reactions requires systematic design of suitable length ODNs, as well as optimization of the distance between the two probes, the reaction conditions, and the workup procedures.

A number of templates (ODNs **12a**–**d**) containing sequences that flank and include codon 248 in exon 7 of the p53 gene were generated. ODN **12b** contained the wild-type genetic sequence, while ODN **12a** contained the mutated G nucleotide associated with cancer. A 12-mer ODN (ODN **11**), which contained an alkyne-modified coumarin at the 5′-terminus and was complementary to the 5′ region of the templates (upstream of the SNP site), was used as the first probe. ODN **4**, which contained an azide group and was complementary to the 3′ region of the templates (upstream of the SNP site), was initially used as the second probe (Figure 4A); there was no nucleotide gap between the ODN **4** and ODN **11** sequences. ODN **4** and ODN **11** were efficiently ligated with a yield of 89% in the presence of the perfectly matched target (ODN **12a**). The "click" ligation followed first-order kinetics ($k_L = 2.88 \times 10^{-3}$ s^{-1}) and was complete within 3 h when the reaction mixture was incubated at 28 °C (Figure S10B, Supporting Information). The probes were unable to discriminate between the perfectly matched ODN **12a** template and the mismatched ODN **12b** template when the "click" reaction was performed at room temperature; under these conditions, ODN **12b** produced a yield of 84% ligated product **18** (Figure S11A, Supporting Information). However, excellent selectivity was achieved when the reaction temperature was increased to 45 °C, which is higher than the T_m of the mismatched duplex (40 °C for ODN **4**-ODN **12a**) (Figure S11B, Supporting Information).

To be compatible with mild conditions, the fluorogenic "click" ligation reaction must show sequence-selectivity at room temperature; therefore, the lengths of the probes were optimized in an attempt to achieve this requirement. Two truncated versions of ODN **4**, namely, ODN **8** (8-mer) and ODN **10** (9-mer), were designed and the T_ms of the DNA duplexes were determined (Table 3). Stable duplexes were formed between the perfectly matched template (ODN **14a**) and ODN **8** or ODN **10**; the T_ms of the duplexes were 37.5 and 44.1 °C, respectively (Table 3). The T_ms of the mismatched duplexes formed between ODN **8** or ODN **10** and templates containing a SNP at codon 248 (ODNs **14b**–**d**) were lower than room temperature (<15 °C for the ODN **8**-ODN **14b**–**d** duplexes and <21.5 °C for the ODN **10**-ODN **14b**–**d** duplexes) (Table 3). These data indicate that both probes could discriminate perfectly matched template (ODN **14a**) from mismatched templates (ODN **14b**–**d**) at room temperature with a ΔT_m of greater than 22 °C.

To avoid steric hindrance between the azide and coumarin moieties, ODN **8** and ODN **10** were designed to be positioned one nucleotide downstream from ODN **11** (Figure 4A). The "click" ligation reaction between ODN **11** and ODN **8** or ODN **10** was performed in the presence of ODNs **12a**–**d** as target templates (Figure 4A). As expected, the ligation reactions were more efficient in the presence of the matched ODN **12a** template (Figure 4B and C, lane 2) than the mismatched ODN **12b**–**d** templates (Figure 4B and C, lanes 3–5); however, the ligation yield of ODN **8** (14.8%; Figure 4B, lane 2) was markedly lower than that of ODN **10** (90.0%; Figure 4C, lane 2), which is possibly due to the relatively low T_m of ODN **8**. In most cases, not all wild-type targets are mutated identically; some targets remain intact while others undergo different mutations. Therefore, the ability of ODN probes to detect a matched template in a mixture of different mismatched

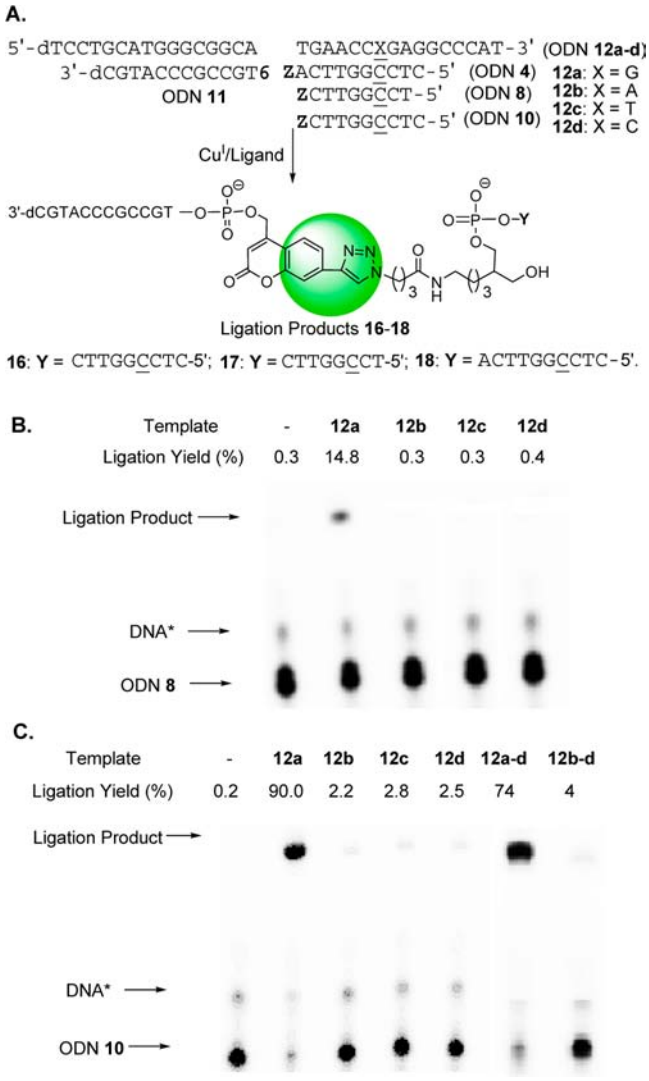


Figure 4. (A) DNA sequences (ODNs **4**, **8**, **10**, **11**, and **12a**–**d**) used in the DNA-templated "click" ligation reaction. (B,C) Phosphorimage autoradiogram of denaturing PAGE analysis of the "click" ligation reactions between 75 nM ODN **11** and 50 nM ODN **8** (B) or ODN **10** (C) (ODN **8** or ODN **10** was labeled with ^{32}P at the 5′-terminus). Reactions were performed in the absence (lane 1) or presence (lanes 2–5) of 75 nM ODN **12a**, **b**, **c**, or **d**; the presence of a mixture of ODNs **12a**–**d** (50 nM each) (lane 6); or the presence of a mixture of ODNs **12b**–**d** (50 nM each) (lane 7). All "click" reactions were performed at room temperature for 3 h in the presence of THPTA, $CuSO_4$, and sodium ascorbate.

dx.doi.org/10.1021/bc4001678 | *Bioconjugate Chem.* 2013, 24, 1226–1234

Table 3. Melting Temperatures of the Duplexes Formed Between Matched and Mismatched Targets of ODN 8 and ODN 10[a]

Duplex	T_m (°C)
5′-dTGAACCGGAG-3′ (ODN **14a**) 3′-dZCTTGGCCT (ODN **8**)	37.5 ± 0.5
5′-dTGAACCAGAG-3′ (ODN **14b**) 3′-dZCTTGGCCT (ODN **8**)	<15
5′-dTGAACCTGAG-3′ (ODN **14c**) 3′-dZCTTGGCCT (ODN **8**)	<15
5′-dTGAACCCGAG-3′ (ODN **14d**) 3′-dZCTTGGCCT (ODN **8**)	<15
5′-dTGAACCGGAG-3′ (ODN **14a**) 3′-dZCTTGGCCTC (ODN **10**)	44.1 ± 0.6
5′-dTGAACCAGAG-3′ (ODN **14b**) 3′-dZCTTGGCCTC (ODN **10**)	21.5 ± 0.5
5′-dTGAACCTGAG-3′ (ODN **14c**) 3′-dZCTTGGCCTC (ODN **10**)	21.2 ± 0.5
5′-dTGAACCCGAG-3′ (ODN **14d**) 3′-dZCTTGGCCTC (ODN **10**)	19.9 ± 0.5

[a]Measured in a buffer containing 0.1 M NaCl, 10 mM potassium phosphate (pH 7), and 4 μM of each single-strand ODN. The position of the mismatch in ODNs **14b–d** of the p53 sequence is underlined.

templates is important for their use in cellular applications. To investigate whether the ODN probes designed in this study were capable of functioning under cellular conditions, the fluorogenic "click" ligation of ODN **10** and ODN **11** was performed in the presence of a mixture of matched (ODN **12a**) and mismatched (ODN **12b–d**) templates. An efficient ligation reaction with a yield of 74% was observed under these conditions (Figure 4C, lane 6), while only a trace amount of ligation product was observed in the presence of a mixture of the mismatched ODNs **12b–d** only (Figure 4C, lane 7).

ODNs **10**, **11**, and **12a** were used to optimize the reaction conditions for the template-directed "click" ligation reaction. Optimization involved varying the ratio of THPTA to $CuSO_4$ (Figure S12A, Supporting Information), the concentration of $CuSO_4$ (Figure S12B, Supporting Information), and the ratio of $CuSO_4$ to sodium ascorbate (Figure S13, Supporting Information). The ratio of THPTA to $CuSO_4$ had a large effect on the ligation efficiency; the optimum ratio was determined to be 20:1. The minimum concentrations of $CuSO_4$ and sodium ascorbate required for an efficient "click" ligation were determined to be 4 mM and 8 mM, respectively. The optimum conditions (80 mM THPTA, 4 mM $CuSO_4$, and 40 mM sodium ascorbate; incubation for 3 h at 25–28 °C) were used in all subsequent "click" ligation reactions and DNA detection procedures.

The rate constant for formation of a ligation product between ODN **10** and ODN **11** (k_L= (2.3 ± 0.3) × 10^{-4} s^{-1}; $t_{1/2}$ = 49.6 min; Figure S14B, Supporting Information) was within the experimental error of that for ODN **4** and ODN **11** (k_L= (2.9 ± 0.4) × 10^{-4} s^{-1}; $t_{1/2}$ = 40.1 min; Figure S10B, Supporting Information). ODN **4** and ODN **11** are positioned immediately adjacent to each other, whereas a single nucleotide gap is present between ODN **10** and ODN **11**. Therefore, these data suggest that a nucleotide gap between the two probes does not affect the ligation reaction. However, the rate of the "click" ligation reaction was approximately four times slower than that of the "click" ICL reaction (k_{ICL} = (1.1 ± 0.2) × 10^{-3} s^{-1}; Figure S7B, Supporting Information). The ODN **11**-ODN **10** ligation product (ODN **16**) was isolated and characterized by MALDI-TOF (m/z = 6830.5 $[M+H]^+$, calcd. m/z = 6831.3; Figure S15, Supporting Information). The fluorescence intensities of ODN **16** (10 μM) and a mixture of unreacted ODN **10** (10 μM) and ODN **11** (10 μM) were measured. The fluorescence intensity was enhanced approximately 11-fold (λ_{em} 415 nm) following formation of the ligation product (Figure 5A). Therefore, a highly efficient fluorogenic "click" ligation reaction capable of single nucleotide discrimination at room temperature was achieved by using a 9-mer ODN probe.

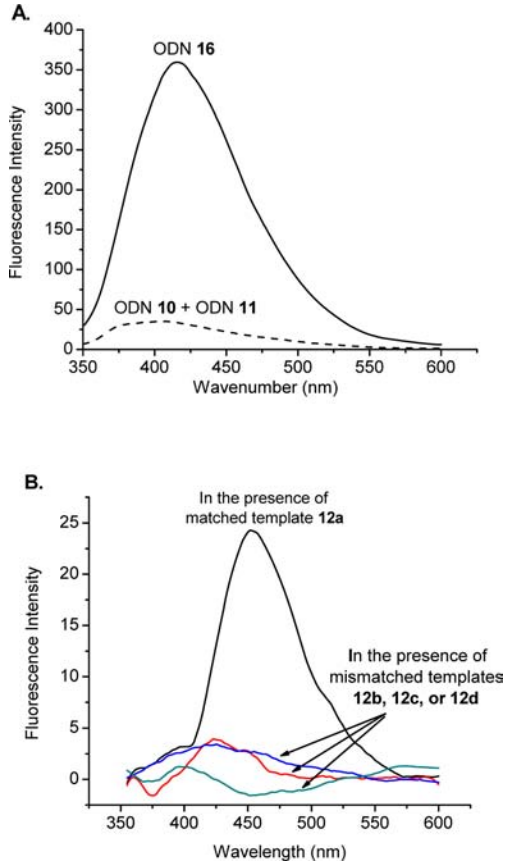


Figure 5. (A) Fluorescence emission spectra of the gel-purified ligation product (ODN **16**; 10 μM) (solid line) and a mixture of unreacted ODN **10** (10 μM) and ODN **11** (10 μM) (dashed line) (λ_{ex} = 330 nm, slit width = 10 nm; λ_{em} = 410 nm, slit width = 10 nm) in water (pH 7) at room temperature. (B) Fluorescence emission spectra of a reaction mixture containing 0.4 μM ODN **10** and 0.4 μM ODN **11** in the presence of different templates (ODNs **12a–d**) in water at the r.t. The catalyst, ligand, and salts were removed by precipitation and by passing the reaction through an Illustra G-25 column. λ_{ex} = 330 nm, slit width = 5 nm; λ_{em} = 440 nm, slit width = 15 nm. The spectra were measured in water (pH 7) at room temperature.

Detection of SNPs in the p53 Tumor Suppressor Gene using a Fluorogenic "Click" Ligation Reaction. Having developed a fluorogenic "click" ligation reaction capable of single nucleotide discrimination, the utility of this method for detecting SNPs was evaluated using wild-type and mutant p53 alleles. The ODN **12a–d** templates were hybridized with equimolar concentrations of ODN **10** and **11**. THPTA, $CuSO_4$, and sodium ascorbate were added to the mixture to initiate the ligation reaction and the samples were incubated at 25 °C for 3 h. After removal of the catalyst, ligand, and salts by

dx.doi.org/10.1021/bc4001678 | Bioconjugate Chem. 2013, 24, 1226–1234

precipitation and by passing the reaction mixture through an Illustra G-25 column, the DNAs were dissolved in water and their concentrations were determined by measuring absorbance at 260 nm with a Varian CARY-100 BIO UV−vis spectrophotometer. The fluorescence intensity of 0.4 μM DNA was measured using a Perkin-Elmer LS55 fluorescence spectrometer. A control reaction was performed under the same conditions in the absence of templates. The measured fluorescence spectra were background corrected to the signal from the control sample. The fluorescent signal was greatly enhanced in the presence of the perfectly matched ODN **12a** target, indicating the occurrence of the fluorogenic "click" ligation reaction (Figure 5B). However, the fluorescent signal was not detectable above background levels in the presence of the mismatched ODN **12b−d** templates. ODN **10** and ODN **11** showed 10−20-fold selectivity (calculated as $F_{matched}/F_{mismatched}$) for the matched versus mismatched target sequences, without the need to remove unreacted probes. Therefore, the fluorogenic "click" ligation reaction can be used for SNP detection under mild conditions and removal of unreacted probes is not essential.

The fluorescence intensity of the ligation product ODN **16** (F = 360) was higher than that of the ICL product ODN **15** (F = 210) at the same concentration (10 μM) (Figure S16, Supporting Information). This result indicates slight fluorescence quenching of the cross-linking product but not the ligation product. Charge transfer processes between nucleobases and fluorophores in duplex DNA quench the fluorescence intensity of the fluorophores.[43] The ICL product exists as a duplex that allows more efficient charge transfer from the coumarin moiety to the nucleobases, which may account for the decreased fluorescence intensity. By contrast, the ligation product is a single-stranded DNA species, which prohibits DNA charge transfer. Overall, the fluorogenic "click" ligation is an excellent chemical reaction for specific DNA sequence detection.

CONCLUSION

This report describes the development of a DNA-templated fluorogenic "click" reaction that employs a nonfluorescent alkyne-modified coumarin moiety. The "click" reaction was used to ligate and cross-link two oligonucleotide strands, which resulted in the formation of an enhanced fluorescent signal. The fluorogenic "click" ligation and cross-linking reactions are sequence-specific and are capable of rapid and easy detection of DNA sequences with single nucleotide discrimination under mild conditions. The use of nonfluorescent DNA probes ensures a low background signal, which improves the sensitivity of the technique. The covalent attachment of two probes via "click" chemistry produces a permanent fluorescent signal, which avoids the need to remove unreacted probes with stringent washing procedures. This approach may be useful for *in vivo* nucleic acid detection, as well as for the construction of fluorogenic nanomaterials. The approach described here could potentially be extended to develop strategies that enable inexpensive, reliable, and accurate SNP analysis without the use of costly equipment and reagents. Further investigations are currently underway to develop a Cu-free fluorogenic "click" reaction and methods for signal amplification.

ASSOCIATED CONTENT

Supporting Information

Synthesis of Compound **6**, NMR spectrum of Compond **7**, ESI-MS of alkylated and azido-functionalized oligonucleotides, MALDI-TOF of DNA duplexes, PAGE analysis of DNA ICL and ligation reactions, and fluorescence spectra.This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +414-229-2989; Fax: 414-229-5530; E-mail: pengx@uwm.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof. David N. Frick for reading the manuscript and for his helpful comments. We also thank J. Craig Blain for measuring the mass spectra of ODNs 4, 5, 8, 10, and 11. We are grateful for financial support from the UWM Research Growth Initiative (RGI101 × 234), the Great Milwaukee Foundation (Shaw Scientist Award), and UWM start-up funds.

REFERENCES

(1) Kanan, M. W., Rozenman, M. M., Sakurai, K., Snyder, T. M., and Liu, D. R. (2004) Reaction discovery enabled by DNA-templated synthesis and in vitro selection. *Nature 431*, 545−549.

(2) Silverman, A. P., and Kool, E. T. (2006) Detecting RNA and DNA with templated chemical reactions. *Chem. Rev. 106*, 3775−3789.

(3) Rajski, S. R., and Williams, R. M. (1998) DNA cross-linking agents as antitumor drugs. *Chem. Rev. 98*, 2723−2795.

(4) Noll, M. D., McGregor Mason, T., and Miller, P. S. (2006) Formation and repair of interstrand cross-links in DNA. *Chem. Rev. 106*, 277−301.

(5) Bando, T., Narita, A., Saito, I., and Sugiyama, H. (2003) Highly efficient sequence-specific DNA interstrand cross-linking by pyrrole/imidazole CPI conjugates. *J. Am. Chem. Soc. 125*, 3471−3485.

(6) Dohno, C., Uno, S. N., and Nakatani, K. (2007) Photoswitchable molecular glue for DNA. *J. Am. Chem. Soc. 129*, 11898−11899.

(7) Di Giusto, D. A., Knox, S. M., Lai, Y., Tyrelle, G. D., Aung, M. T., and King, G. C. (2006) Multitasking by multivalent circular DNA aptamers. *ChemBioChem. 7*, 535−544.

(8) Xu, Y., Karalkar, N. B., and Kool, E. T. (2001) Nonenzymatic autoligation in direct three-color detection of RNA and DNA point mutations. *Nat. Biotechnol. 19*, 148−152.

(9) Abe, H., Wang, J., Furukawa, K., Oki, K., Uda, M., Tsuneda, S., and Ito, Y. (2008) A reduction-triggered fluorescence probe for sensing nucleic acids. *Bioconjugate Chem. 19*, 1219−1226.

(10) Barany, F. (1991) Genetic disease detection and DNA amplification using cloned thermostabile ligase. *Proc. Natl. Acad. Sci. U.S.A. 88*, 189−193.

(11) Landegren, U., Kaiser, R., Sanders, J., and Hood, L. (1988) A ligase-mediated gene detection technique. *Science 241*, 1077−1080.

(12) Haff, L. A., Wilson, C. G. M., Huang, Y., Benton, B. K., Bond, J. F., Stern, A. M., Begley, R. F., and Coull, J. M. (2006) DNA-programmed chemistry in rapid homogeneous assays for DNA and protein targets. *Clin. Chem. 52*, 2147−2148.

(13) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective 'ligation' of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596−2599.

(14) Nwe, K., and Brechbiel, M. W. (2009) Growing applications of "click chemistry" for bioconjugation in contemporary biomedical research. *Cancer Biother. Radiopharm. 24*, 289−302.

(15) Kocalka, P., El-Sagheer, A. H., and Brown, T. (2008) Rapid and efficient DNA strand cross-linking by click chemistry. *ChemBioChem. 9*, 1280−1285.
(16) Pujari, S. S., Xiong, H., and Seela, F. (2010) Cross-linked DNA generated by "bis-click" reactions with bis-functional azides: site independent ligation of oligonucleotides via nucleobase alkynyl chains. *J. Org. Chem. 75*, 8693−8696.
(17) Xiong, H., and Seela, F. (2011) Stepwise "click" chemistry for the template independent construction of a broad variety of cross-linked oligonucleotides: influence of linker length, position and linking number on DNA duplex stability. *J. Org. Chem. 76*, 5584−5597.
(18) Xiong, H., and Seela, F. (2012) Cross-linked DNA: site selective "click" ligation in duplexes with bis-azides and stability changes caused by internal cross-links. *Bioconjugate Chem. 23*, 1230−1243.
(19) Xiong, H., Leonard, P., and Seela, F. (2012) Construction and assembly of branched Y-shaped DNA: "click" chemistry performed on dendronized 8-aza-7-deazaguanine oligonucleotides. *Bioconjugate Chem. 23*, 856−870.
(20) Pujari, S. S., and Seela, F. (2012) Cross-linked DNA: propargylated ribonucleosides as "click" ligation sites for bifunctional azides. *J. Org. Chem. 77*, 4460−4465.
(21) Shelbourne, M., Chen, X., Brown, T., and El-Sagheerz, A. H. (2011) Fast copper-free click DNA ligation by the ring-strain promoted alkyne-azide cycloaddition reaction. *Chem. Commun. 47*, 6257−6259.
(22) Kumar, R., El-Sagheer, A. H., Tumpane, J., Lincoln, P., Wilhelmsson, L. M., and Brown, T. (2007) Template-directed oligonucleotide strand ligation, covalent intramolecular DNA circularization and catenation using click chemistry. *J. Am. Chem. Soc. 129*, 6859−6864.
(23) Peng, X., Li, H., and Seidman, M. (2010) A template-mediated click−click reaction: PNA−DNA, PNA−PNA (or peptide) ligation, and single nucleotide discrimination. *Eur. J. Org. Chem. 22*, 4194−4197.
(24) Chouikhi, D., Barluenga, S., and Winssinger, N. (2010) Clickable peptide nucleic acids (cPNA) with tunable affinity. *Chem. Commun. 46*, 5476−5478.
(25) Seela, F., and Sirivolu, V. R. (2008) Pyrrolo-dC oligodeoxynucleotides bearing alkynyl side chains with terminal triple bonds: synthesis, base pairing and fluorescent dye conjugates prepared by the azide-alkyne "click" reaction. *Org. Biomol. Chem. 6*, 1674−1687.
(26) Droumaguet, C. L., Wang, C., and Wang, Q. (2010) Fluorogenic click reaction. *Chem. Soc. Rev. 39*, 1233−1239.
(27) Sivakumar, K., Xie, F., Cash, B. M., Long, S., Barnhill, H. N., and Wang, Q. (2004) A fluorogenic 1,3-dipolar cycloaddition reaction of 3-azidocoumarins and acetylenes. *Org. Lett. 6*, 4603−4606.
(28) Gierlich, J., Burley, G. A., Gramlich, P. M. E., Hammond, D. M., and Carell, T. (2006) Click chemistry as a reliable method for the high-density postsynthetic functionalization of alkyne-modified DNA. *Org. Lett. 8*, 3639−3642.
(29) Seela, F., Sirivolu, V. R., and Chittepu, P. (2008) Modification of DNA with octadiynyl side chains: synthesis, base pairing, and formation of fluorescent coumarin dye conjugates of four nucleobases by the alkyne−azide "click" reaction. *Bioconjugate Chem. 19*, 211−224.
(30) Berndl, S., Herzig, N., Kele, P., Lachmann, D., Li, X., Wolfbeis, O. S., and Wagenknecht, H. A. (2009) Comparison of a nucleosidic vs non-nucleosidic postsynthetic "click" modification of DNA with base-labile fluorescent probes. *Bioconjugate Chem. 20*, 558−564.
(31) Beatty, K. E., Xie, F., Wang, Q., and Tirrell, D. A. (2005) Selective dye-labeling of newly synthesized proteins in bacterial cells. *J. Am. Chem. Soc. 127*, 14150−14151.
(32) Beatty, K. E., Liu, J. C., Xie, F., Dieterich, D. C., Schuman, E. M., Wang, Q., and Tirrell, D. A. (2006) Fluorescence visualization of newly synthesized proteins in mammalian cells. *Angew. Chem., Int. Ed. 45*, 7364−7367.
(33) Neef, A. B., and Schultz, C. (2009) Selective fluorescence labeling of lipids in living cells. *Angew. Chem., Int. Ed. 48*, 1498−1500.
(34) Ming, X., and Seela, F. (2012) A nucleobase discriminating pyrrolo-dC click adduct designed for DNA fluorescence mismatch sensing. *Chem.—Eur. J. 18*, 9590−9600.
(35) Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
(36) Zhou, Z., and Fahrni, C. J. (2004) A fluorogenic probe for the copper(I)-aatalyzed azide-alkyne ligation reaction: modulation of the fluorescence emission via $^3(n, \pi^*)$-$^1(\pi, \pi^*)$ inversion. *J. Am. Chem. Soc. 126*, 8862−8863.
(37) Choi, H. Y., and Chi, D. Y. (2003) Nonselective bromination-selective debromination strategy: selective bromination of unsymmetrical ketones on singly activated carbon against doubly activated carbon. *Org. Lett. 5*, 411−414.
(38) Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science 287*, 2007−2010.
(39) Mancuso, T., Aguilar, F., Pescarolo, M. P., Clerico, L., Russo, P., and Parodi, S. (1997) Mutation frequencies at codon 248 of the p53 tumour suppressor gene are not increased in colon cancer cell lines with the RER^+ phenotype. *Nucleic Acids Res. 25*, 3643−3648.
(40) Peng, X., and Greenberg, M. M. (2008) Facile SNP detection using bifunctional, cross-linking oligonucleotide probes. *Nucleic Acids Res. 36*, e31.
(41) Chan, T. R., Hilgraf, R., Sharpless, K. B., and Fokin, V. V. (2004) Polytriazoles as copper(I)-stabilizing ligands in catalysis. *Org. Lett. 6*, 2853−2855.
(42) Kanan, M. W., Rozenman, M. M., Sakurai, K., Snyder, T. M., and Liu, D. R. (2004) Reaction discovery enabled by DNA-templated synthesis and in vitro selection. *Nature 431*, 545−549.
(43) Garo, F., and Häner, R. (2012) 2,1,3-Benzothiadiazole-modified DNA. *Eur. J. Org. Chem. 14*, 2801−2808.