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Reversible modification of oligodeoxynucleotides: click reaction at phosphate group and alkali treatment

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ABSTRACT

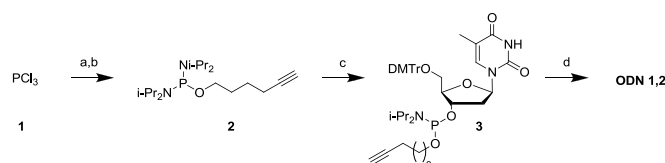
We characterized click reaction between oligodeoxynucleotides (ODNs) possessing acetylene groups at the phosphate unit and azide compounds. Cu(I)-catalyzed cycloaddition proceeded efficiently to form the corresponding functional ODNs. The resulting ODNs could be converted into ordinary ODNs by treatment with aqueous methylamine. The present method successfully achieved a reversible modification of ODNs.

Functional oligodeoxynucleotides (ODNs) provide considerable potential for a wide range of genetic technologies such as gene diagnostics and therapy.¹⁻⁴ In past years, various attempts have been made to develop new procedures for the preparation of ODNs possessing diverse functionalities⁵⁻⁷ by solid-phase or enzyme-assisted synthetic methods.⁸⁻¹³ The postsynthetic-modification method is one of the most useful technologies for the preparation of functionalized ODNs. Functional groups are usually incorporated into ODNs by means of several linkers possessing primary alkyl amines, thiols and formyl groups after the synthesis and deblocking of the ODNs. This protocol generally furnishes various functions to ODNs with less effort than the preparation of reagents for incorporation during synthesis. Modification protocols have provided various useful ODNs, however, almost all protocols were based on irreversible chemical reactions and thereby the uses of ODNs for genetic studies were limited. To expand the versatility of artificial ODNs, the development of reversible modification protocol is imperative.

Click chemistry has been used for modification of biomolecules, including proteins and nucleic acids.¹⁴⁻²² In particular, the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction between acetylene and azide groups has good regioselectivity and reaction efficiency,^{15,17,18} and thereby is recognized as a practical and bioorthogonal tool for the functionalization of DNA. Herein, we report a novel chemical modification of ODNs by a click reaction at the phosphate group. Acetylene-containing phosphate groups were incorporated into ODNs, and the click reactions were performed in the presence of azide compounds. The cycloaddition reaction proceeded efficiently to produce the corresponding functional ODNs. We also found that treatment with aqueous methylamine solution led to removal of the functional group to form ordinary ODNs without modification. Thus, the present protocol offers a reversible modification method for ODNs.

As shown in Scheme 1, we have synthesized ODNs possessing acetylene terminuses at the phosphate unit. Trichlorophosphine 1 was treated with *N,N*-diisopropylamine and coupled with 5-hexyn-1-ol. Then, given 2 was incorporated into

the DNA strand via phosphoramidite 3,²³ using a DNA synthesizer.²⁴ The structure of the synthesized ODNs, as confirmed by MALDI-TOF mass spectrometry, is summarized in Figure 1.^{25,26}



Scheme 1. Reagents and conditions: (a) *N,N*-diisopropylamine; (b) 5-Hexyn-1-ol, iPr_2NEt ; (c) 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine, DMAP, iPr_2NEt , 65% (3 steps) (d) automated DNA synthesis.

We initially conducted click reactions in the presence of $CuSO_4$ and tris-(benzyltriazolyl)methylamine (TBTA) using sodium ascorbate as a reducing agent in aqueous solution containing 20% *t*-BuOH. The cycloaddition was performed between ODN 1 possessing one acetylene group and an azide compound, azide-PEG-NH₂, and was monitored by reversed-phase HPLC. The appearance of a new peak in Figure 2c was attributable to the formation of the corresponding ODN 3, into which the ethylene glycol derivative possessing an amino terminus was incorporated, according to the MALDI-TOF mass spectroscopy results. The chemical yield for cycloaddition was estimated to be 80%.²⁷ We also conducted a click reaction using ODN 2 possessing two acetylene units in one strand. A similar reaction proceeded and the doubly modified ODNs were produced with a yield of 90%. Thus, the present reaction would be applicable to multimodification of ODNs. In separate experiments, we conducted a click reaction of ODN 1 in the presence of $CuSO_4$ and sodium ascorbate but without TBTA. The cycloaddition under these conditions was unsuccessful, resulting in the decomposition of starting material ODN 1. This result indicates that stabilization of Cu(I) and protection of DNA degradation by TBTA is indispensable for the present click reaction at the phosphate group, similar to the case in previous reports.¹³

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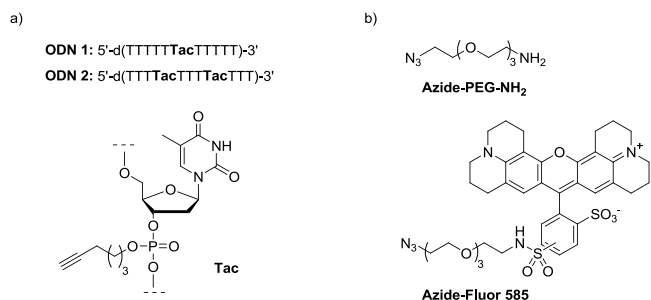


Figure. 1 (a) Sequences and structure of oligodeoxynucleotides used in this study. (b) Structures of the incorporated functional molecules into DNA strand by click reaction.

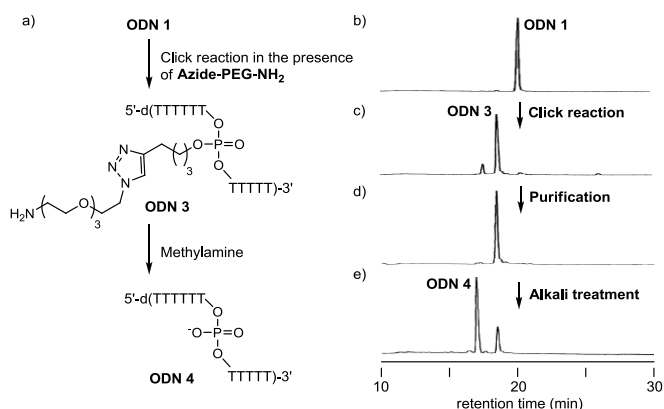


Figure. 2 (a) Click reaction of ODN 1 in the presence of Azide-PEG-NH₂ and removal of functional group by treatment with methylamine to form ordinary DNA strand. (b,c,d,e) HPLC profiles for click reaction and treatment by methylamine. The elution peaks were monitored at 260 nm wavelength. Click reaction between ODN 1 (50 μM) and Azide-PEG-NH₂ (300 μM) was performed in the presence of CuSO₄ (10 μM), Na ascorbate (50 μM) and TBTA (10 μM), while removal of functional group was carried out in the presence of 40% methylamine in aqueous solution. (b) before the reaction; (c) after click reaction for 2 h at 40 °C; (d) after purification of ODN 3 (e) after treatment of ODN 3 with methylamine for 20 h at 40 °C.

It is well-known that the methyl phosphotriester linkage in modified DNA is labile to alkali treatment to form a regular phosphodiester linkage. This reaction characteristic of the phosphate group prompted us to demonstrate the removal of a functional group incorporated by the present click reaction. We conducted a treatment of ODN 3 with aqueous methylamine solution for 20 h at 40 °C. Representative HPLC profiles of the reaction are shown in Figures 2d and 2e. While prompt degradation of ODN 3 was observed under these conditions, the phosphotriester group was efficiently converted to phosphodiester group, resulting in the formation of an ordinary DNA strand, ODN 4. The formation of ODN 4 was confirmed by MALDI-TOF mass spectrometry and overlap injection of an authentic sample in HPLC analysis. The yield for the formation of ODN 4 was 63%. These results strongly indicate that ODNs modified by a click reaction at the phosphate unit can be converted to regular ODNs without any modification by treatment with an alkali such as methylamine.

In light of the above reaction characteristics, further attempts were made to apply the present system to fluorescence labeling of DNA. A click reaction between ODN 1 and a fluorophore containing azide group (azide-Fluor 585) was conducted in the

presence of CuSO₄, TBTA and sodium ascorbate, and monitored by HPLC (Figure 3). After incubation for 2 h at 40 °C, two new products (ODNs 5a and 5b) were produced with yields of 14% (ODN 5a) and 51% (ODN 5b), respectively. The products were purified by HPLC and then incorporation of the fluorescent molecule into both products was identified by MALDI-TOF mass spectrometry and visual confirmation of fluorescence emission (Figure 3d). The formation of two products was attributed to the regioisomers of azide-Fluor 585. We also conducted removal of the fluorescent functional group from ODN 5 by treatment with aqueous methylamine and confirmed the formation of unmodified ODN 4 in good yield (Figure 3c).²⁸ Thus, reversible fluorescence labeling of ODNs was achieved by the present system using click chemistry and alkali treatment.

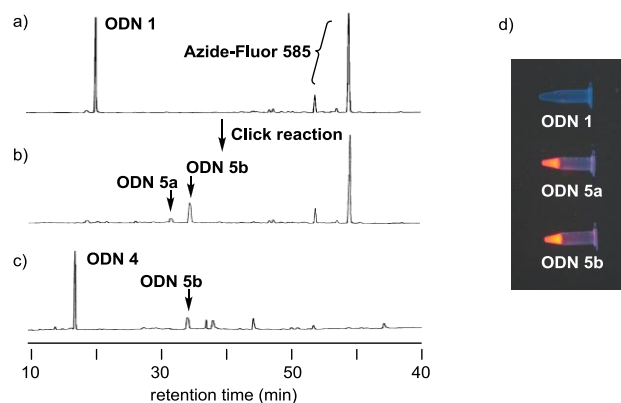


Figure. 3 (a,b) HPLC profiles for click reaction between ODN 1 (50 μM) and Azide-Fluor 585 (500 μM). The elution peaks were monitored at 260 nm wavelength. Click reaction was performed in the presence of CuSO₄ (10 μM), Na ascorbate (50 μM) and TBTA (10 μM) (a) before the reaction; (b) after click reaction for 2 h at 40 °C; (c) HPLC profile for removal of fluorescent functional group from purified ODN 5b by treatment with 40% methylamine for 23 h at 40 °C; (d) Visualization of fluorescence emission of product (ODN 5a and ODN 5b, Excitation at 365 nm).

In conclusion, we have synthesized novel ODNs possessing acetylene units at the phosphate group and demonstrated their click reaction in the presence of functional azide compounds. Cycloaddition between the acetylene unit and the azide group occurred smoothly, leading to formation of the corresponding functionalized ODNs. It is striking that treatment by aqueous methylamine solution resulted in removal of functional group at the phosphate unit to form an ordinary phosphodiester group. Thus, ODNs modified by the click reaction could be converted to native ODNs.

The present modification system would be widely applicable to various biological tools including modification of 3'-terminal of the strand,²⁹ reversible labeling and solid-phase synthesis of DNA. Further attempts for functionalization of DNA are now underway.

References and notes

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24. The cleavage of ODNs from solid support was carried out with 50 mM potassium carbonate in methanol for 20 h at ambient temperature.
25. The ODNs used in this study formed stable duplex with their complementary strand (ODN 6: 5'-d(AAAAAAAAAAAA)-3'). Melting temperatures (T_m) were 25.1, 21.1, 25.1, 24.1, 26.6 and 25.6 °C for ODN 1/ODN 6, ODN 2/ODN 6, ODN 3/ODN 6, ODN 4/ODN 6, ODN 5a/ODN 6 and ODN 5b/ODN 6, respectively.
26. The UV spectra of ODN 1 and ODN 3 were shown in Figure S1 (Supplementary Material).
27. The yield of click reaction was calculated from concentrations of ODNs, which were quantified by HPLC.
28. The yield for the formation of ODN 4 was estimated to be 47%.
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