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Click Chemistry as a Reliable Method for the High-Density Postsynthetic Functionalization of Alkyne-Modified DNA

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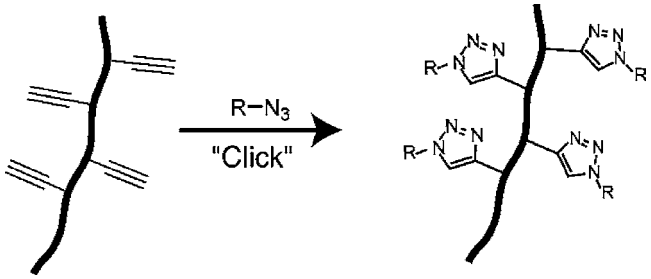
ABSTRACT



We report the development of the Cu(I)-catalyzed Huisgen cycloaddition (click) reaction for the multiple postsynthetic labeling of alkyne-modified DNA. A series of alkyne-modified oligodeoxyribonucleotides (ODNs) of increasing alkyne density were prepared, and the click reaction using various azide labels was investigated. Complete high-density conversion was observed for ODNs containing up to six consecutive alkyne functions. Compatibility of the click conditions with long DNA strands was shown using a PCR product obtained with an alkyne-modified primer.

The development of fast, simple, and reliable DNA sequencing and detection methods is important for life science research and the diagnosis of pathogenic and genetic disorders.[1−3] A variety of methods are now available for the sequencing of specific DNA strands or genetic variants thereof.[4] However, protocols that rely on the incorporation and detection of fluorescently tagged nucleoside building blocks have been by far the most sensitive, with single-molecule sensitivity achieved within milliseconds.[5] Unfortunately, the enzymatic replacement of each natural building block with a fluorescently tagged analogue is a challenging exercise, often requiring highly modified protocols.[1,2,6,7] An alternative method is the incorporation of small chemical reporter groups into particular genes that can be further functionalized (termed postsynthetic functionalization).[8] Such a methodology requires a modular protocol in which appropriately modified nucleoside building blocks are incorporated into DNA strands either by an enzymatic process (e.g., the PCR) or via solid-phase synthesis. As a second

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requirement, this post-synthetic functionalization method must be a highly efficient and specific process, resulting in the quantitative conversion of reporter groups into labeled products. Of the handful of chemical motifs that possess the required attributes for use as chemical reporter groups,[8] azides and terminal alkyne functions have been shown to be elegantly suited for biomolecular ligation via the Cu(I)-mediated Huisgen cycloaddition reaction (click chemistry).[9] Indeed, preliminary investigations by our group have demonstrated the power of incorporating reporter group modified nucleosides to direct metal deposition for DNA detection.[10]

We now report the construction of modified oligodeoxyribonucleotides (ODNs) bearing alkyne reporter groups in high density and the development of a click reaction protocol which now enables loading of DNA in high yield with a variety of molecular labels. This two-step process involving the initial chemical of enzymatic incorporation of an alkyne−nucleoside building block and postsynthetic functionalization can be used to decorate DNA for identification or isolation according to the nature of the probe.

To evaluate whether click chemistry[11,12] would be a useful postsynthetic method for high-density labeling of DNA, the modified uridine nucleosides **1** and **2** were prepared and incorporated into a series of 16-mer ODNs via their corresponding phosphoramidites.[13] To circumvent potential steric problems with the high-density labeling of ODNs containing the alkyne **1**, we also prepared the nucleoside **2**. The alkyne function in **2** is separated from the uridine base by a flexible spacer. Incorporation of building blocks **1** and **2** into 16-mer strands via solid-phase DNA synthesis proceeded smoothly albeit with a slight alteration in the phosphoramidite coupling protocol (Table 1). The compatibility of the click

Table 1. ODN Series Comprising **1** or **2**

	X = 1
ODN-1	5'-GCG CTG TXC ATT CGC G
ODN-2	5'-GCG CTG XXC ATT CGC G
ODN-3	5'-GCG CXG TXC AXT CGC G
ODN-4	5'-GCG CXX XXX XGT CGC G
	Y = 2
ODN-5	5'-GCG CTG TYC ATT CGC G
ODN-6	5'-GCG CTG YYC ATT CGC G
ODN-7	5'-GCG CYG TYC AYT CGC G
ODN-8	5'-GCG CYY YYY YGT CGC G
ODN-9	5'-TTA ATT GAA TTC GAT TYG GGC CGG AYT TGT TTC
ODN-10	5'-GCA GGC YTCA YGC CAG AAT TAC CAG AAG

reaction was then investigated via the coupling of the ODN series in Table 1 with azides **3**−**5**. Azides **3**−**5** were chosen

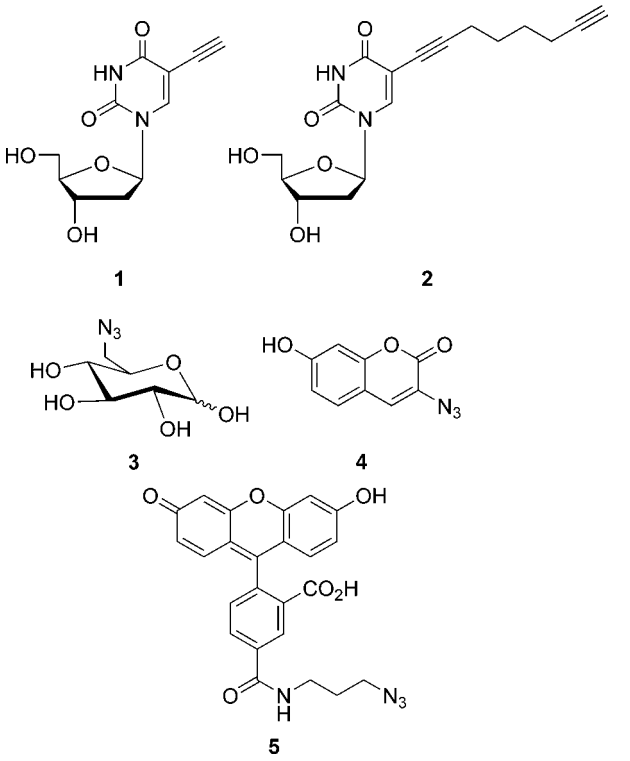

Figure 1.

as they represent a small selection of desirable labels. Azidosugar **3** is a semiprotected aldehyde used for selective Ag staining;[10] coumarin azide **4** fluoresces only after triazole formation,[14] and fluorescein azide **5** is a strongly fluorescent molecule used in a variety of biophysical applications.[15]

In the presence of excess azide, a Cu(I) salt, and ODN-**1** or ODN-**5** (i.e., ODNs comprising one click site), a range of adducts corresponding to strand breaks were observed, suggesting that the original click procedure was not amenable to high-density functionalization of DNA. However, using the Cu(I)-stabilizing ligand (tris(benzyltriazolylmethyl)-amine),[9,14,16−18] full conversion of both ODN-**1** and ODN-**4** to their respective triazole products was observed using azides **3**−**5** with no apparent degradation. This finding was consistent with previous results[19,20] which conclude that the Cu(I)-stabilizing ligand protects biomolecules from unwanted aqueous Cu(I)-mediated chemistry, such as •OH production.[21]

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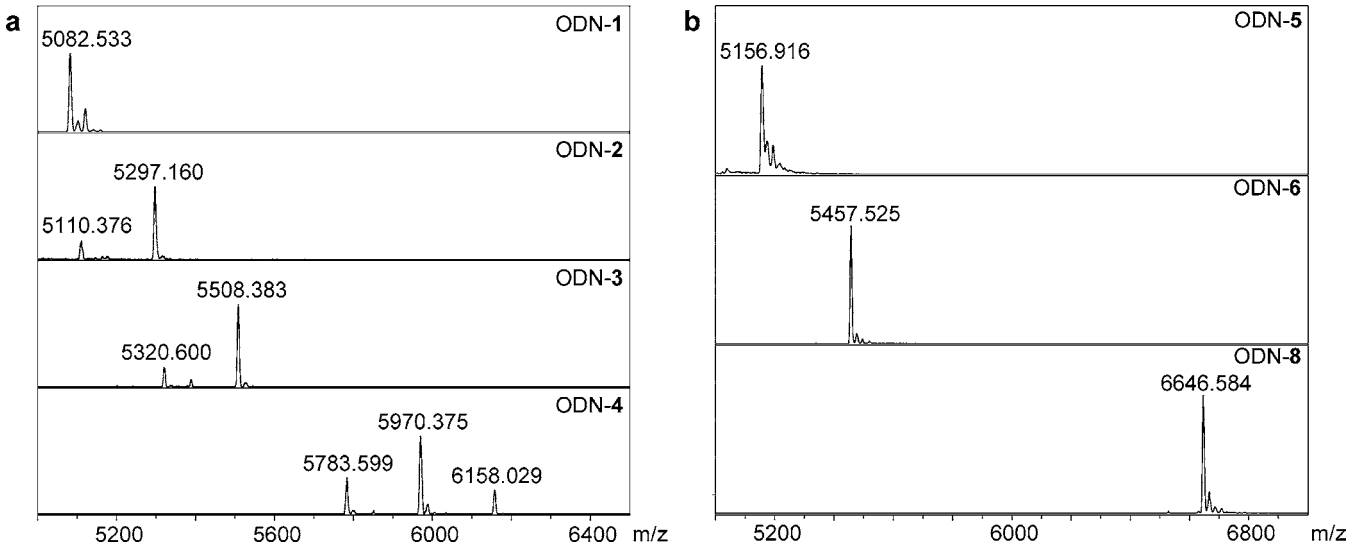

Figure 2. MALDI-TOF spectra of: (a) click reaction performed with azide **3** and ODNs comprising alkyne **1** with one (ODN-**1**, MW = 4872), two (ODN-**2**, MW = 4882), three (ODN-**3**, MW = 4892), and six (ODN-**4**, MW = 4902) alkyne functions and (b) click chemistry performed with azide **3** and ODNs comprising alkyne **2** with one (ODN-**5**, MW = 4952), two (ODN-**6**, MW = 5042), and six (ODN-**8**, MW = 5379) alkyne functions.

The rigid alkyne series ODN-**1**−ODN-**4** was then assayed using azides **3**−**5**. Investigation of the click reaction using the sugar azide **3** and ODN-**1** revealed complete conversion (Figure 1a, ODN-**1**, *m*/*z* 5082 [M + Na^+]) by MALDI-TOF analysis; however, when the alkyne density was increased to two adjacent alkyne click sites (Figure 2a, ODN-**2**), an additional small amount of the monoclick product (*m*/*z* 5110 [M + Na^+]) was observed in addition to the fully clicked product (*m*/*z* 5297). This result was also consistent with the click reaction between **3** and the ODN containing three alkyne functions (ODN-**3**) separated by two bases each. A minor amount of the two-clicked adduct (*m*/*z* 5320 [M + Na^+]) was obtained in addition to the three-clicked adduct (*m*/*z* 5508) as the major product. These findings were also supported by the reaction with ODN-**4** containing six adjacent alkynes. The major click product then corresponded to only a five-clicked adduct (*m*/*z* 5970 [M + 2Na^+]) in addition to minor products corresponding to a four- (*m*/*z* 5784 [M + 3Na^+]) and six- (*m*/*z* 6158 [M + Na^+]) clicked adduct, respectively. Therefore, it became apparent that the steric shielding of the alkyne by the DNA backbone (ODN-**1**−ODN-**4**) was contributing to the less than optimal labeling yields. In contrast to the ODN-**1**−ODN-**4** series, full conversion was observed for ODN-**5**, ODN-**6**, and ODN-**8** containing the flexible alkyne according to MALDI-TOF analysis (Figure 2b).

Switching to the nonfluorescent coumarin azide **4** provided the added benefit of forming a highly fluorescent click product. Both ODN series (ODN-**1**−ODN-**3** and ODN-**5**−ODN-**8**) were assayed against azide **4** and revealed a clicking scenario similar to that observed for azide **3**: the rigid alkyne series (ODN-**1**−ODN-**3**) produced less than quantitative conversion to the fluorescent click product, whereas the flexible alkyne series (ODN-**5**−ODN-**8**) produced fully labeled products. With the flexible alkyne spacer nucleoside **2**, it is therefore possible to achieve highly reliable, complete high-density functionalization of ODNs.

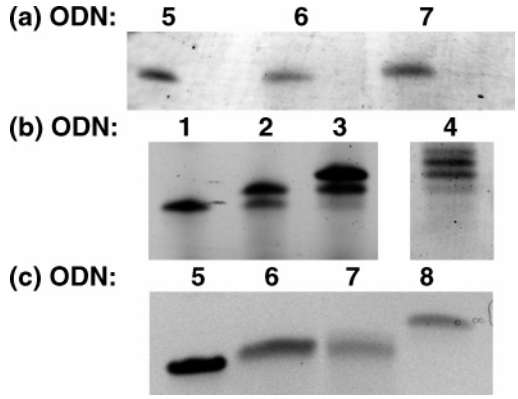

Figure 3. Gel electrophoresis of click reactions. (a) Click reactions of ODNs containing **2** with coumarin azide **4**; detection using a 460 nm cutoff filter. (b) Click reactions of the ODN series containing the nucleoside **1** and the fluorescein azide **5**. (c) Click reactions of the ODN series containing the nucleoside **2** and the fluorescein azide **5**.

Gel electrophoresis studies of coumarin-labeled ODN products (ODN-**5**−ODN-**7**) are depicted in Figure 3a.

When click chemistry was performed using the fluorescein azide **5**, the resulting products were easily observed by the difference in electrophoretic mobility as a consequence of a much larger increase in molecular weight (Figure 3b,c). As expected, in the case of the ODN-**1**−ODN-**4** series, only partial conversion was observed (Figure 3b). For two click

sites (lane 2) and three click sites (lane 3), the product mixtures are clearly visible. The click reaction between **5** and ODN-**4** in particular revealed a range of products (Figure 3b, lane 4). Consistent with previous click studies using the ODN-**5**−ODN-**8** series, full conversion was observed for all strands by gel electrophoresis (Figure 3c). Lanes 1−4 show complete conversion of one, two, three, and six click sites.

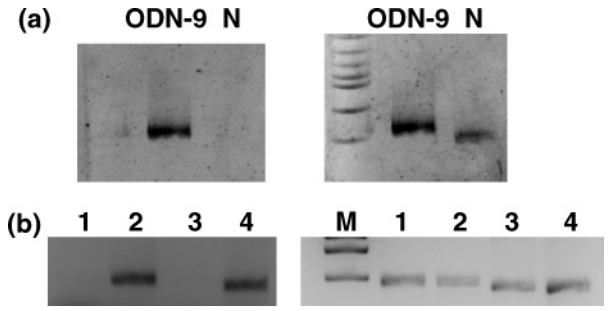

Figure 4. Click reactions on the PCR product using fluorescein azide **5**. (a) Click on 300 bp PCR fragment comprising ODN-**9** (lane 1: 100 bp marker; lane 2: alkyne DNA (primer ODN-**9**); lane 3: control with unmodified DNA; N = natural). The right gel is stained with SYBR Green II. (b) Agarose gels of a click on an 800 bp PCR product using ODN-**10** (lane 2) and a 900 bp PCR product using ODN-**9** (lane 4). Lanes 1 and 3 are controls using unmodified primers. The right gel was run with ethidium bromide.

To investigate whether click chemistry can be utilized to label long DNA fragments, for a labeling method based on click chemistry, the PCR primers ODN-**9** and ODN-**10** containing two click sites were synthesized and subsequently used in PCR to amplify a range of PCR products from two different plasmid templates. Melting point analysis of ODN-**9** revealed no destabilization of the DNA strand due to the two modifications.

The resulting amplicons comprise two alkyne moieties at one end of the double strand. The purified DNA was then treated with azide **5** using the CuBr/ligand method.[16−18] Gel electrophoresis of the reaction product showed only a single band corresponding to a fluorescein labeled click product (Figure 4a, lane 2, and Figure 4b, lanes 2 and 4). An unmodified DNA fragment treated identically showed no fluorescence (Figure 4a, lane 3, Figure 4b, lanes 1 and 3). No sign of DNA degradation was observed when staining the gels with SYBR Green II or ethidium bromide in either case. The same results were obtained on PCR fragments up to 2000 base pairs.[13]

In summary, click chemistry is a simple and robust method for the conversion of alkyne-modified DNA into labeled products. Using the alkyne nucleoside **2**, the high-density reliable modification of all alkyne sites can be achieved. The method can even be employed to modify long DNA fragments obtained by PCR without DNA cleavage. Efforts are now underway to investigate the utility of click chemistry in the functionalization of specific genes of several hundred to several thousand base pairs in length.

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Supporting Information Available: Information concerning the preparation of **1**−**5**, the synthetic ODNs, and ODN clicking efficiency (MALDI-TOF and electrophoretic data). PCR conditions and primer sequences and clicking on a 1000 and 2000 bp fragment. This material is available free of charge via the Internet at http://pubs.acs.org.

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