1 Postsynthetic DNA Modification via the Copper-Catalyzed Azide-Alkyne Cycloaddition

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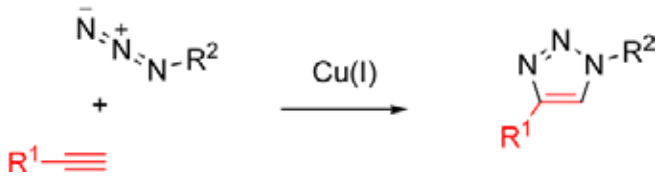
1.1 Abstract

The attachment of labels onto DNA is of utmost importance in many areas of biomedical research. It also proves valuable in the construction of DNA-based functional nanomaterials. Today, the most widespread methods in use for the conjugation of DNA to functional molecules suffer from low conversion efficiencies or the susceptibility of the reactant functional groups to side reactions. The Cu(I)-catalyzed Huisgen cycloaddition (click reaction) has recently been added to the repertoire of DNA labeling methods, allowing the virtually unlimited functionalization of both small synthetic oligonucleotides and large gene fragments with unprecedented efficiency. The click reaction yields the labeled polynucleotides in very high purity after a simple precipitation step. The reviewed technology is currently changing the way of how functionalized DNA strands are generated cost-efficiently in high quality for their application in molecular diagnostics systems and nanotechnological research.

1.2 Introduction

The 1,3-dipolar cycloaddition between azides and alkynes -the Huisgen reaction[1]- has recently found a tremendous number of novel applications (~1000 publications) after the discovery that the reaction can be efficiently catalyzed by Cu(I).[2] This copper-catalyzed form of the reaction (Huisgen-Meldal-Sharpless reaction) is presently the most prominent example of a class of reactions named click reactions. These reactions are, according to Sharpless' definition, characterized by high yields, mild reaction conditions and by the fact that they tolerate a broad range of functional groups. Typically, the reactions require simple or no workup and purification of the product. For reasons of simplicity, the Cu(I)-catalyzed alkyne azide cycloaddition (CuAAC) discussed here will be referred to as click reaction.



The most important characteristic of the click reaction is its unique bioorthogonality, as both azide and terminal alkyne functional groups are generally not present in natural systems. A wide range of biomolecules has thus been labeled to date, including peptides,[3] proteins,[4] polysaccharides[5] and even an entire virus.[6] The use of this method for DNA modification has been somewhat delayed by the fact that copper ions damage DNA, typically yielding

strand breaks.[7] As these problems have now been overcome by the use of a Cu(I)-stabilizing ligand (TBTA),[8] click chemistry is in an excellent position to take over as the state-of-the-art methodology to label and modify DNA.

In a variety of applications such as molecular diagnostics, DNA needs to bear labels like fluorescent dyes for detection of the DNA molecule. In addition biotin is often attached to DNA to facilitate DNA purification or most recently carbohydrates have been used to functionalize DNA in order to allow selective silver deposition or to set up carbohydrate microarrays. Two general strategies exist for the labeling of oligonucleotides called presynthetic and postsynthetic labeling (Figure 1.1). Presynthetic labeling denotes that the nucleotide monomers already carry the desired label before DNA synthesis, deprotection and purification. Postsynthetic labeling requires the introduction of a small reactive moiety into DNA, which can be coupled to the label later on, after completion of DNA synthesis.

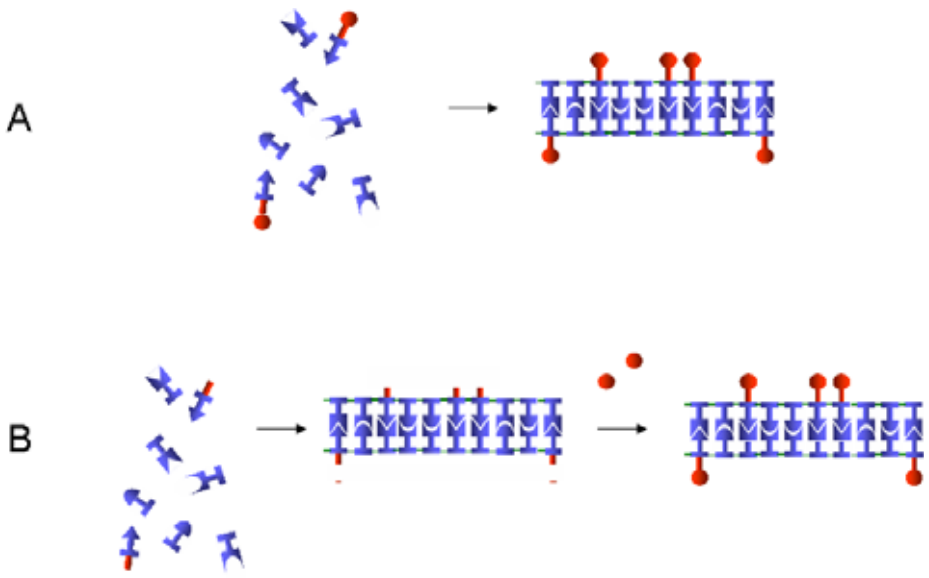
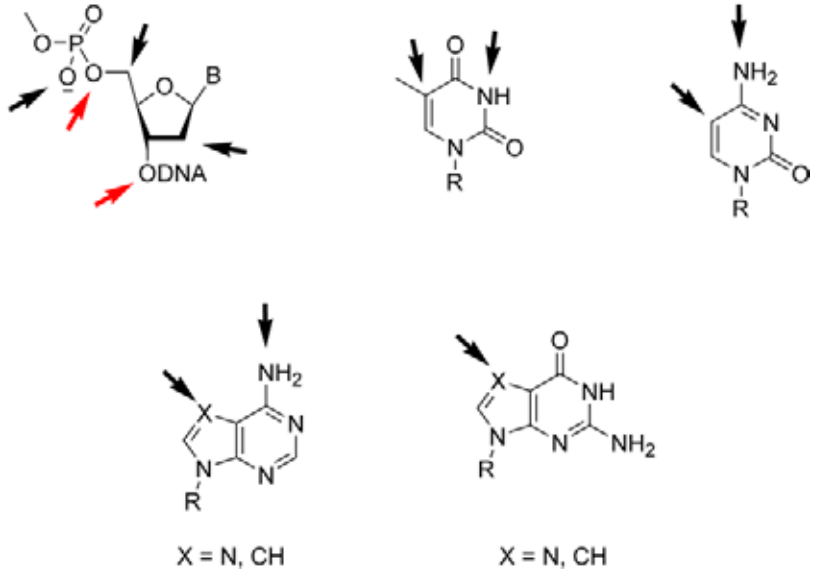


Figure 1.1 Schematic representation for presynthetic (A) and postsynthetic labeling (B).

In the presynthetic strategy (A in Figure 1.1) a phosphoramidite bearing the desired label is synthesized, which needs to be stable enough to survive the harsh conditions employed during solid phase synthesis (acidic, alkaline and oxidative conditions) and deprotection (strongly alkaline conditions) of the DNA strand. Additionally, the P(III) phosphoramidite group, which is susceptible to oxidation to the corresponding P(V) phosphoramidate, needs to be stable in the presence of the label. This method thus excludes the use of labels with reactive functional groups or inherent base or acid-sensitivity. Alternatively, the label can be prepared as the corresponding triphosphate using DNA polymerases to insert the label during primer extension or PCR. This method is limited to those modifications which are accepted as substrates by polymerases and there are often steric limitations.

In the postsynthetic labeling strategy (B in Figure 1.1), a small reactive group is introduced into DNA, which can then be conjugated to the desired functional molecule in a selective manner after DNA synthesis and deprotection. Presently, the most widely utilized method for

postsynthetic labeling is to prepare DNA bearing an amino group (amino modifier), which reacts with an active ester functional group (e.g. NHS) of the desired modification.[9] Sensitive or reactive moieties can be introduced in this way, but the strategy is highly dependent on the selectivity and efficiency of the postsynthetic modification step. This strategy allows for a higher degree of modularity, because changing the label does not require a novel nucleotide synthesis as in the case of presynthetic labeling. However, the low coupling yields and the need for intensive oligonucleotide purification by HPLC after the labeling is limiting this approach making labeled oligonucleotides very expensive compounds. Nucleobases which carry the reactive amino group can also be used as triphosphates for the incorporation into DNA by either primer extension or PCR.[10]



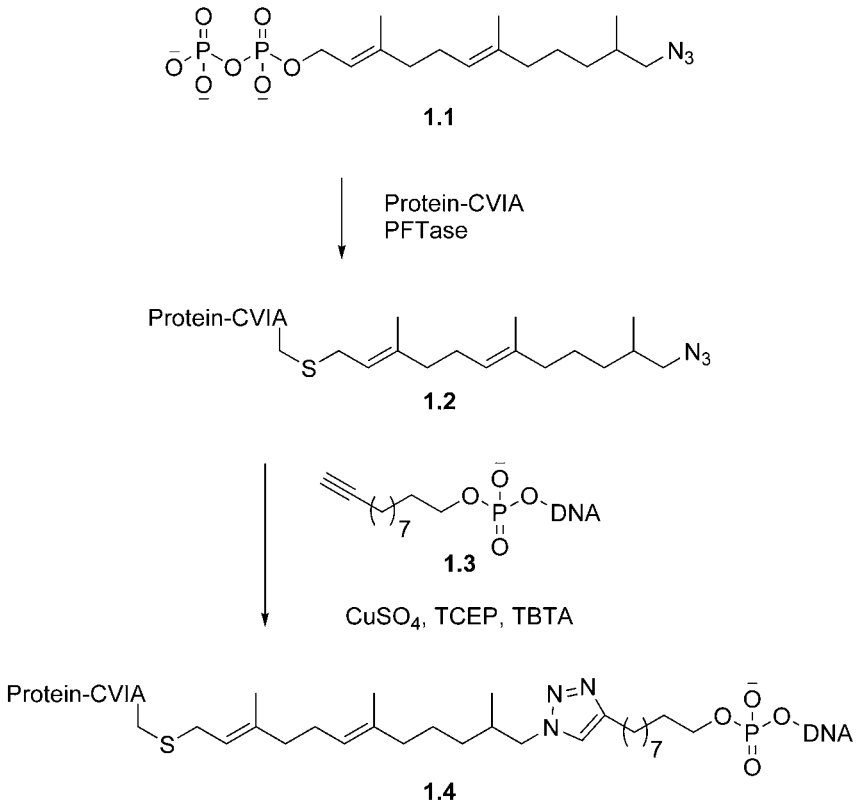
Scheme 1.1 Overview over the different positions that have up to now been used for the attachment of azides or alkynes onto DNA. Red arrows indicate attachment points for terminal modification only.

Click chemistry offers solutions to these issues. The chemistry is clean, high yielding and operates at mild conditions. In order to allow oligonucleotide modification by the click reaction, however, DNA building blocks are required which carry either alkyne or azide functional groups. As depicted in Scheme 1.1, alkyne or azide functionalities have already been attached to many different positions on DNA. In addition a number of non-nucleosidic alkyne/azide DNA modifiers have been prepared.[11, 12] In this minireview we summarize the various approaches to modify oligonucleotides with the click reaction. In Sections 1.2 to 1.6 we describe the click chemistry applications of DNA strands prepared by solid-phase synthesis. In Section 1.7, we summarize click chemistry in combination with enzymatic strategies.

1.3 Preparation of Surface Immobilized DNA with Click Chemistry

The first example of a successful click chemistry modification of DNA[a] used a 5′-alkylamino-modified DNA strand, which was reacted with succinimidyl 5-azidovalerate to display an azide group at the 5′-terminus.[13, 14] Thermal (not Cu(I)-catalyzed) cycloaddition with an alkyne-modified fluorescein furnished the reaction product in excellent yield, but as a regioisomeric mixture of the 1,4- and 1,5-triazoles. These mixtures are typical for the Huisgen cycloaddition without Cu(I) catalysis. The direct incorporation of azides into synthetic DNA strands is intrinsically difficult because the azide group reacts with the P(III) of the phosphoramidite group in a Staudinger reduction.[15] However, the two step procedure for attaching the azide to the DNA strand allowed the subsequent attachment of the DNA to a glass surface for a sequencing by synthesis approach.[16] An alternative approach for the immobilization of DNA on surfaces using click chemistry has employed an alkyne group attached to the 5′-OH of a DNA strand, which reacted with azide modified gold surfaces.[17] Even simpler is the approach of Reinhoudt et al.. This group pressed a polydimethylsiloxane (PDMS) stamp, which was covered with alkyne modified DNA, directly onto an azide-terminated glass slide to achieve the click reaction under pressure without any Cu(I) catalyst added. [12]

1.4 Preparation of DNA-Protein Conjugates with Click Chemistry

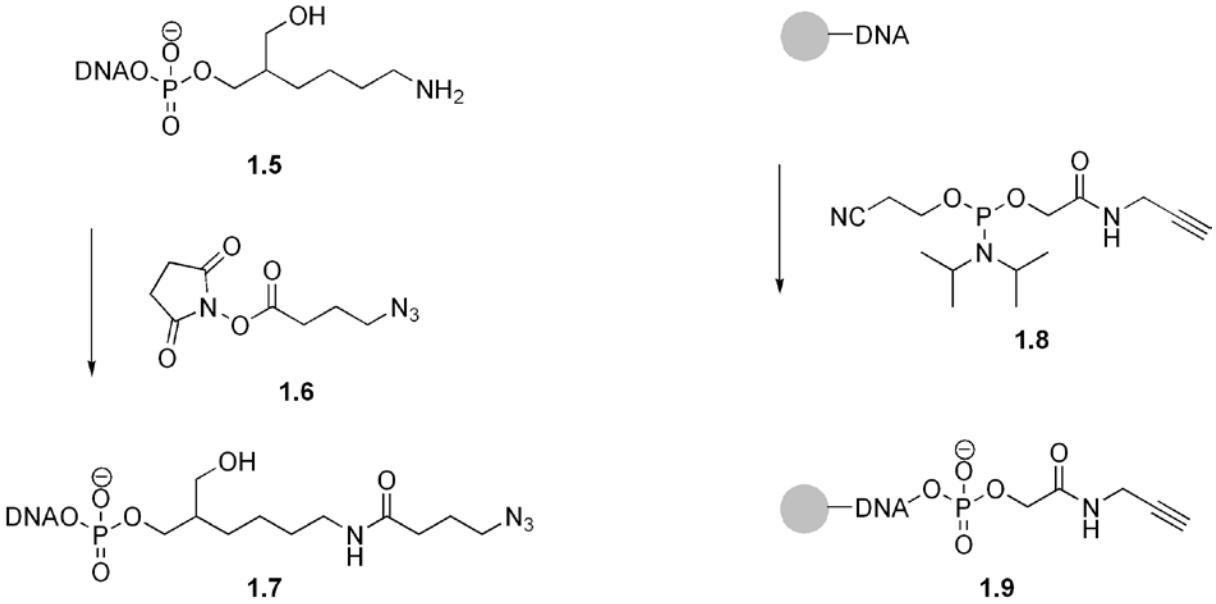


Scheme 1.2 Synthetic strategy for DNA-protein conjugates.

[a] At this point the cycloaddition was performed thermally without Cu(I) catalysis, presumably due to the DNA degrading effect of free Cu(I) species in aqueous solutions. After publication of a Cu(I) stabilizing ligand, which accelerates the click reaction, most of the following work makes use of the Cu(I) catalyzed version of the Huisgen cycloaddition. Nonetheless the work by Ju et al. is seen as the prototypical example of click chemistry on DNA.

Click chemistry has been successfully utilized for the preparation of complex DNA-protein conjugates.[18] Distefano et al. attached the specific tetrapeptide CVIA to green fluorescent protein (GFP). To this tag the authors specifically attached an azide modified isoprenoid diphosphate with the help of the enzyme protein farnesyltransferase (PFTase, Scheme 1.2).[19] The obtained azide bearing protein was then reacted with a single stranded oligonucleotide (ssODN), carrying an alkyne attached to a 5′-terminal phosphate group. In order to test whether the DNA sequence can still be addressed despite the presence of the protein, a counter strand containing a Texas Red label was successfully hybridized to the DNA strand attached to the protein. Using this construct, the group successfully built up nanoscale DNA tetrahedra,[20] which were finally decorated with up to four GFPs. The methodology is currently being exploited for the preparation of protein modified DNA-nanostructures.

1.5 Preparation of Circularized and Branched DNA Structures Using Click Chemistry



Scheme 1.3 Building blocks for the synthesis of circularized and catenated DNA.

Click chemistry has vastly simplified the synthesis of circularized and catenated DNA structures.[15, 21] Brown et al. have devised a strategy to label a ssODNs with both a 5′-terminal alkyne and a 3′-terminal azide.[22] The azide was generated as described by Ju et al.[13] by coupling of succinimidyl 4-azidobutyrate to an amino linker at the DNA yielding the required 3′-terminal azide (**1.7** in Scheme 1.3). The alkyne group, in contrast, was introduced at the 5′-terminus by terminating the solid phase synthesis with the propargylated phosphoramidite **1.8**. Such a doubly modified DNA strand reacts under high dilution conditions with itself in a click reaction to give a cyclic ssODN. When the complementary ODN, which also carries an azide and an alkyne group, is hybridized to this cyclic ODN, it can in turn also be cyclized to yield a covalently catenated dsDNA. In this reaction, the first ODN templates the click reaction of the second DNA strand which reduces side reactions. The catenated DNA showed a vastly increased resistance to exonuclease degradation.