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Nucleotidyl transferase assisted DNA labeling with different click chemistries

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ABSTRACT

Here, we present a simple, modular and efficient strategy that allows the 3′-terminal labeling of DNA, regardless of whether it has been chemically or enzymatically synthesized or isolated from natural sources. We first incorporate a range of modified nucleotides at the 3′-terminus, using terminal deoxynucleotidyl transferase. In the second step, we convert the incorporated nucleotides, using either of four highly efficient click chemistry-type reactions, namely copper-catalyzed azide-alkyne cycloaddition, strain-promoted azide-alkyne cycloaddition, Staudinger ligation or Diels-Alder reaction with inverse electron demand. Moreover, we create internal modifications, making use of either ligation or primer extension, after the nucleotidyl transferase step, prior to the click reaction. We further study the influence of linker variants on the reactivity of azides in different click reactions. We find that different click reactions exhibit distinct substrate preferences, a fact that is often overlooked, but should be considered when labeling oligonucleotides or other biomolecules with click chemistry. Finally, our findings allowed us to extend our previously published RNA labeling strategy to the use of a different copper-free click chemistry, namely the Staudinger ligation.

INTRODUCTION

'Click chemistry', a class of highly efficient, bioorthogonal chemical reactions (1), has become very popular for labeling nucleic acids and other biomolecules (2,3). Besides the well-known copper-catalyzed azide-alkyne cycloaddition (CuAAC) (4,5), often simply referred to as 'click chemistry', alternative reactions have been described that circumvent the use of toxic copper and reducing agents. Examples are the strain-promoted azide-alkyne cycloaddition (SPAAC) (6), also dubbed 'copper-free click chemistry', and Staudinger ligation (7), as well as the Diels-Alder reaction with inverse electron demand (DAR_{inv}) (8). All of the above-mentioned chemistries have been exploited by us or others, to label RNA (9–21) and DNA (3,11,22–30). DNA labeling is required, e.g. to generate fluorescent, biotinylated, spin-labeled or nanoparticle-labeled probes for detection and purification (31–35), labeled functional DNAs such as DNAzymes (36,37) and is of great use in nanotechnology (38). For click-labeling of DNA, the reactive moieties are generally introduced co-synthetically either chemically or by template-dependent polymerases (11,22,25–28). The only available fully post-synthetic 'click'-labeling methods for DNA use methyltransferases to decorate DNA with 'clickable' adenosine analogs (30). 5′-terminal labeling can be achieved easily using a chemically synthesized primer bearing a modification, e.g. a reactive handle (24,29). On the other hand, 3′-terminal DNA labeling methods often rely on terminal deoxynucleotidyl transferase (TdT), which adds non-templated nucleotides to DNA 3′-termini (39,40). TdT has been used to incorporate a range of radioactive and non-radioactive labels into DNA (32,38–41). Surprisingly, although it would constitute a simple, modular and widely applicable labeling method, the direct incorporation of 'clickable' nucleotides into DNA by TdT has been restricted to a variation of the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (42), where DNA strand-breaks in cells are detected by incorporation of 5-ethynyl-deoxyuridine (5-*E*-dU), conversion with an azido-dye and fluorescence detection (https://www.lifetechnologies.com/order/catalog/product/C10245 archived at http://www.webcitation.org/6YcrIwgCX). Therefore, we aimed at incorporating different nucleotides carrying modifications at the base or sugar moieties (Figure 1, Table 1, Supplementary Table S1). These modifications were not confined to the small azide- and alkyne-modifications, so that larger, sterically more demanding functional groups, such as dibenzo-azacyclooctyne (DIBAC) (43) and norbornene were also incorporated (Figure 1B, C), to allow for a wider spectrum of click reactions (CuAAC, SPAAC, Staudinger ligation and DAR_{inv}; Figure 1B) to be performed. As in our previous RNA labeling strategy (9) we investigated whether further manipulations (ligations or primer extensions) can be

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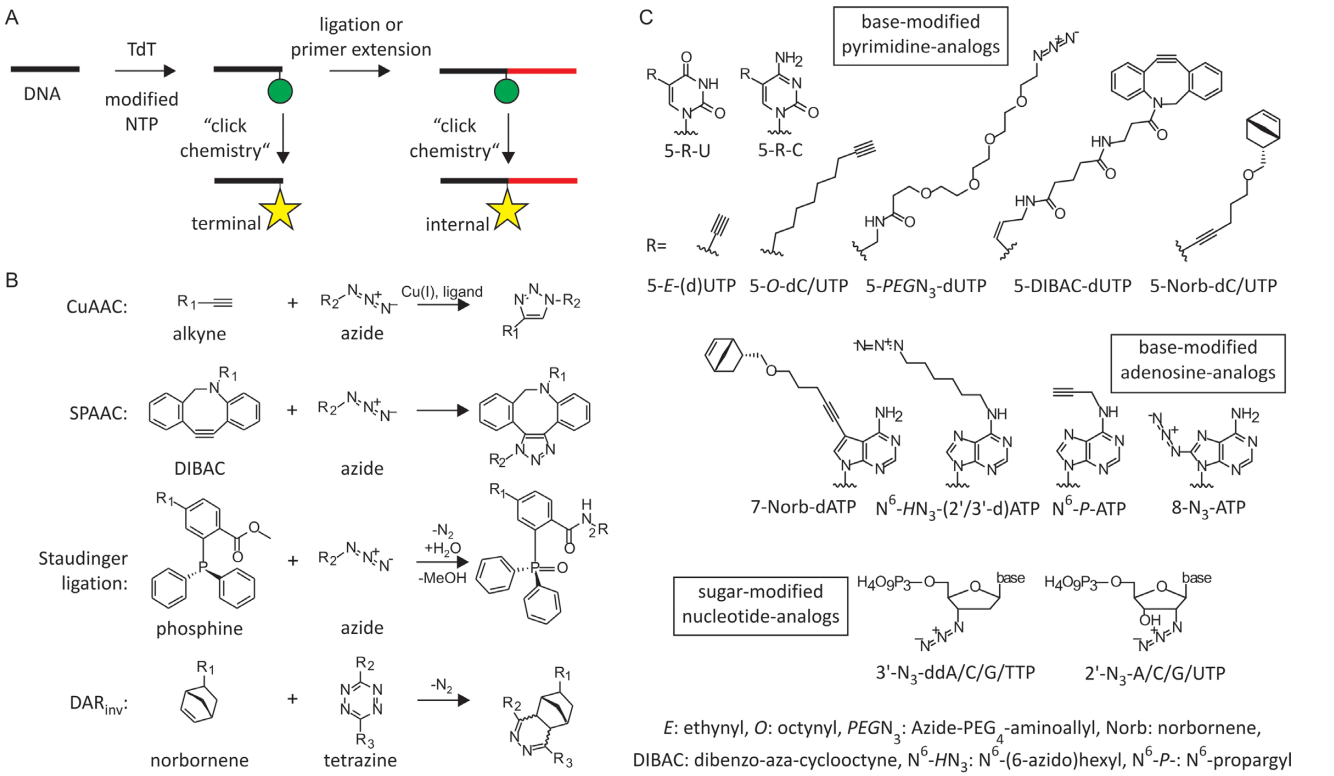

Figure 1. (**A**) Strategy for terminal or internal DNA labeling using nucleotidyl transferases and click chemistry. (**B**) Overview of click reactions used in this work. TdT: Terminal deoxynucleotidyl transferase; NTP: nucleoside triphosphate; CuAAC: copper-catalyzed azide-alkyne cycloaddition; SPAAC: strain-promoted azide-alkyne cycloaddition; DAR_{inv}: Diels-Alder reaction with inverse electron demand. (**C**) Overview of modified nucleotides (more details in Table 1 and Supplementary Table S1).

used to convert certain terminal modifications into internal ones (Figure 1A). We further compared the reactivity of different azido-modified nucleotides in CuAAC, SPAAC and Staudinger ligation to help researchers choose the modified nucleotide and labeling chemistry that will suit their experiments best in the future.

MATERIALS AND METHODS

Oligonucleotides used in this study are given in Supplementary Table S2. Radiolabeling and gel-based analyses, including quantification of bands by ImageQuant program were performed as previously described (9). Settings for fluorescent scans are given in Supplementary Table S3. Enzymes used in this study were from Thermo Scientific unless mentioned otherwise.

Tailing reactions with TdT

For TdT-reactions, DNA was combined with the respective nucleotides in 1x terminal nucleotidyl transferase buffer [Thermo Scientific; 25 mM Tris-HCl (pH 7.2), 200 mM potassium cacodylate, 0.01% (v/v) Triton X-100, 1 mM $CoCl_2$] and TdT (1 U/μl). Unless stated otherwise, tailing reactions were performed at 1 μM DNA concentrations. Nucleotide concentrations were 1 mM for initial tests, but were varied between 1 μM and 1 mM for later reactions, as indicated. Reactions were performed for 90 min or overnight (12-17 h) at 37°C and stopped by heating to 70°C for 10 min. For detection, non-labeled DNA1/2/3 was spiked with 0.01 μM to 0.05 μM radiolabeled DNA1/2/3 and non-labeled DNA4 was spiked with 1 μM fluorescently labeled DNA4 (DNA4-6-FAM). At 1 μM concentration, pure DNA4-6-FAM was employed. When needed, DNA was purified by precipitation with isopropanol in the presence of 0.3 M sodium acetate (pH 5.5).

For TdT tailing of dsDNA, DNA2 was pre-annealed with reverse complement DNA2′. As DNA2′ is equally a substrate for TdT, DNA concentrations were adjusted to 0.5 μM per strand, in order to provide equal amounts (total: 1 μM) of 3′-termini for ss and ds DNA.

Click reactions at the 3′-terminus

All click reactions at DNA 3′-termini were performed with tailed DNA2 and/or DNA3 in sodium phosphate buffer (pH 7; 50 mM for CuAAC, SPAAC and DAR_{inv}; 12.5 mM for Staudinger ligation).

For CuAAC of alkyne-containing DNA biotin azide or Alexa Fluor 647 azide (250 μM; Life Technologies) was added to purified DNA (250 nM) tailed with alkyne-containing nucleotides (TdT-reaction: 1 μM DNA, 10 μM 5-*E*-UTP, 5-*O*-dUTP, or N^6-*P*-ATP, or 100 μM 5-*E*-dUTP). Alternatively, Cy5 azide (500 μM; Jena Bioscience) was added to non-purified TdT mixture (final concentration in CuAAC: 250 nM DNA, 2.5 μM 5-*O*-dUTP). All CuAAC reactions were performed in presence of 100 μM $CuSO_4$, 500 μM Tris-(3-hydroxypropyltriazolylmethyl)-

Table 1. Overview of modified nucleotides

Modified nucleotides: (commercial) full name	Abbreviation	Functional group
2′-N_3-2′-dA/C/G/UTP	2′-N_3-A/C/G/UTP	Azide
3′-N_3-2′,3′-ddA/C/G/TTP	3′-N_3-ddA/C/G/TTP	Azide
8-N_3-ATP	8-N_3-ATP	Azide
Azide-PEG_4-aminoallyl-dUTP	5-*PEG*N_3-dUTP	Azide
N^6-(6-azido)hexyl-3′-dATP	N^6-*H*N_3-3′-dATP	Azide
N^6-(6-azido)hexyl-ATP	N^6-*H*N_3-ATP	Azide
N^6-(6-azido)hexyl-dATP	N^6-*H*N_3-2′-dATP	Azide
5-C8-alkyne-dC/UTP	5-*O*-dC/UTP	Alkyne (octyne)
5-ethynyl-(d)UTP	5-*E*-(d)UTP	Alkyne (ethyne)
N^6-propargyl-ATP	N^6-*P*-ATP	Alkyne (propyne)
5-DBCO-dUTP	5-DIBAC-dUTP	DIBAC
5-norbornene-dC/UTP (22)	5-Norb-dC/UTP	Norbornene
7-norbornene,7-deaza-dATP (22)	7-Norb-dATP	Norbornene

Full (commercial) name and abbreviation used in this article are given. Structures can be found in Figure 1C.

amine (THPTA) (44) and 1 mM sodium ascorbate at 37°C for 2 h.

For SPAAC of DIBAC-containing DNA Cy5 azide (25 μM) was added to non-purified TdT mixture (TdT-reaction: 1 μM DNA, 10 μM 5-DIBAC-dUTP; final concentration in SPAAC: 250 nM DNA, 2.5 μM 5-DIBAC-dUTP) and incubated at 65°C for 20 min.

For DAR_{inv} Cy5 tetrazine (25 μM) was added to non-purified TdT mixture (TdT-reaction: 1 μM DNA, 10 μM Norb-dA/C/UTP; DAR_{inv}: 250 nM DNA, 2.5 μM Norb-dNTP) and incubated at 37°C for 2 h.

Azide-containing DNA [DNA2/3, spiked with the respective radiolabeled DNA; TdT-reacted overnight at 2 μM (3′-N_3-ddATP; 5-*PEG*N_3-dUTP) or 4 μM (2′-N_3-ATP; 8-N_3-ATP; N^6-*H*N_3-3′-dATP) DNA concentration with 4 μM (5-*PEG*N_3-dUTP), 10 μM (N^6-*H*N_3-ATP), 100 μM (2′-N_3-ATP; 8-N_3-ATP) or 1 mM (3′-N_3-ddATP) NTP] was purified (250 nM final) and reacted at variable concentrations of the respective reaction partner (2.5 μM to 2 mM) in CuAAC, SPAAC and Staudinger ligation. For CuAAC, DNA was incubated with Alexa Fluor 647 alkyne (Life Technologies), $CuSO_4$ (100 μM), THPTA (500 μM) and sodium ascorbate (1 mM) at 37°C for 2 h, for SPAAC with DIBAC-Fluor-488 (Jena Bioscience) at 65°C for 20 min, and for Staudinger ligation with DyLight 488-phosphine (Thermo Scientific) at 60°C for 2 h and all products were purified by ethanol precipitation. Product formation (verified by appearance of a fluorescent signal) was quantified ratiometrically for single-tailed DNA, based on radioactive signals.

Internal modification of DNA

For internal labeling of DNA, DNA2 was TdT-reacted with 5-*E*-UTP or N^6-*P*-ATP (100 μM DNA, 100 μM NTP) at 37°C overnight. To prepare internally alkyne-modified DNA2, alkyne-tailed DNA2 was either used as a primer in primer extension or as a ligation fragment in a splinted ligation. To later remove the template/splint (DNA5/6) after either reaction, the 5′-terminus was phosphorylated by T4 PNK [100 μM DNA, 1x T4 DNA Ligase buffer (Thermo Scientific; 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8 at 25°C), 0.5 U/μl T4 PNK], so that 5′-monophosphate-specific λ-exonuclease could be used for digestion of DNA5/6. To avoid misligation of DNA5/6 and ligation fragment DNA7, DNA was 3′-blocked by TdT reaction with ddGTP (100 μM DNA, 1 mM ddGTP, 90 min) and precipitated prior to phosphorylation. Primer extension was performed by performing 5 cycles of a polymerase chain reaction (PCR)-like reaction [5 μM tailed DNA2, 5 μM blocked and phosphorylated DNA5/6, 3 mM $MgCl_2$, 0.5 mM dNTPs and ∼0.25 U/μl lab-prepared Taq Polymerase in 1x Taq polymerase buffer (Rapidozym); denaturation: 94°C, 1 min, annealing: 60°C, 1 min; extension: 72°C, 1 min]. Splinted ligation was performed by first annealing tailed DNA2 with DNA5/6 and DNA7 by heating to 90°C for 30 s and cooling to room temperature for 5 min, adding all other components after this step [final concentrations: 10 μM DNA2, 22.5 μM DNA5/6, 25 μM blocked and phosphorylated DNA7, 50 μM ATP, 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1.5 U/μl T4 DNA ligase] incubating at 37°C for 4 h and heating to 80°C for 10 min. DNA5/6 and DNA7 were optionally removed from reaction mixtures to obtain pure, ligated/extended ssDNA by adding λ-exonuclease (0.25 U/μl for primer extension or 0.5 U/μl for ligation; New England Biolabs) directly into the reaction mixture and incubating at 37°C for 1 h, followed by 80°C for 10 min. DNA was purified by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.5). Purified DNA (ds or ss) was subjected to CuAAC (1 μM DNA, 500 μM biotin azide, 500 μM $CuSO_4$, 2.5 mM THPTA, 5 mM sodium ascorbate) at 50°C for 2 h and reactions were purified by ethanol precipitation.

Staudinger ligation with RNA

2′-N_3-U-functionalized RNA1 was prepared as previously described (9), using 10 μM to 50 μM RNA concentration and 2 h incubation time with yeast PAP. Non-labeled RNA1 was doped with radiolabeled RNA1. N_3-functionalized RNA was purified by ethanol precipitation and incubated with DyLight 488 phosphine for 2 h or overnight (15 h) at 37°C, at DNA concentrations between 40 nM and 50 μM, and phosphine concentrations between 20 μM and 2 mM.

For internal labeling, RNA2 was modified with 2′-N_3-G, ligated to RNA3, using splint DNA8 and purified including DNase I-digest as previously described (9). Purified, ligated RNA (∼1 μM ligation product) was subjected to

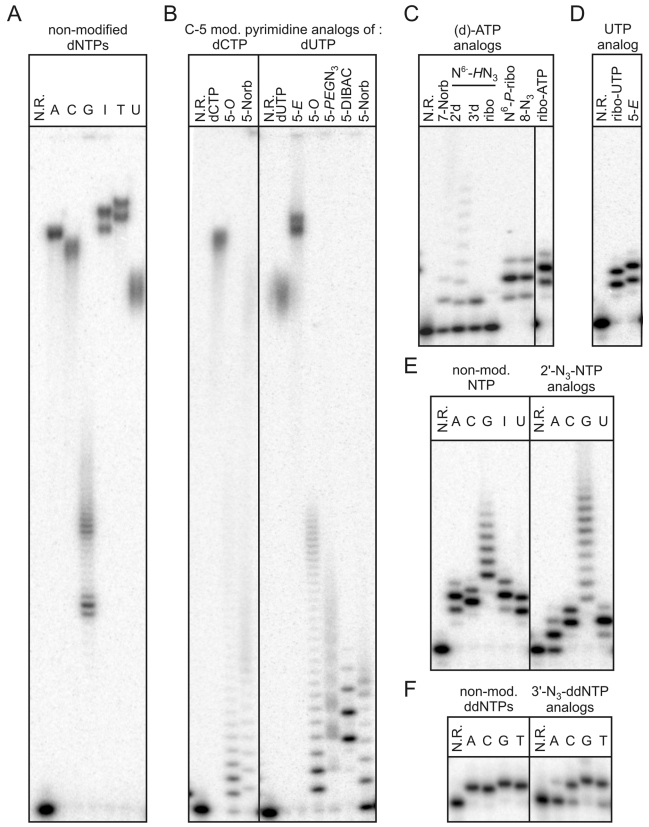

Figure 2. Incorporation of nucleotides by TdT at standard conditions (1 mM nucleotide, 1 μM DNA1, 90 min incorporation). (**A**) Non-modified dNTPs, (**B**) C5-modified dCTP and dUTP analogs, (**C**) base-modified (d)ATP analogs, (**D**) modified UTP analog (5-*E*-UTP), (**E**) non-modified ribo-NTPs and 2′-N_3-NTPs, (**F**) non-modified ddNTPs and 3′-N_3-ddNTPs. Analysis by 15% sequencing PAGE. Phosphor imaging of radioactivity is shown. N.R.: no reaction control. Abbreviations: *E*: ethynyl, *O*: octynyl, Norb: norbornene, *PEG*N_3: azide-PEG_4-aminoallyl, *H*N_3: 6-azidohexyl, *P*: propargyl.

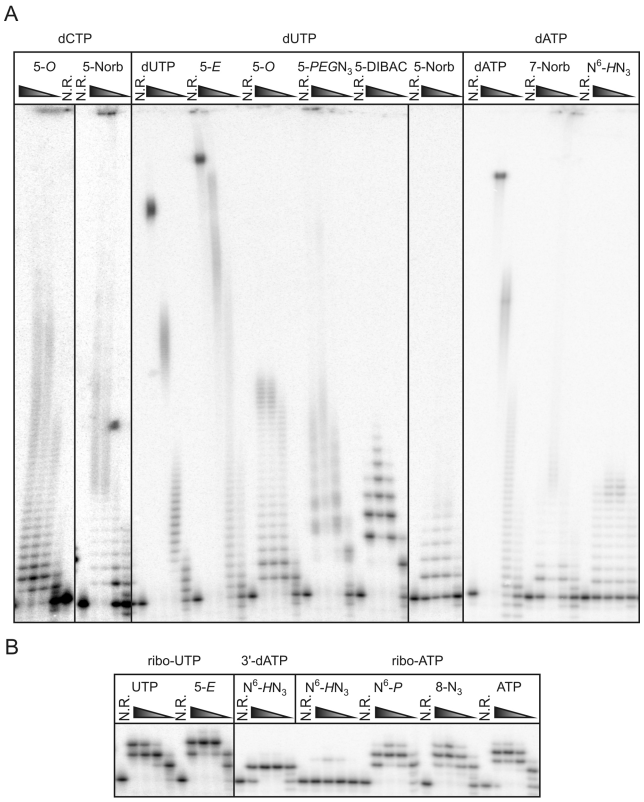

Figure 3. Effect of a reduction in nucleotide concentration for various nucleotides and their base-modified analogs. (**A**) dNTPs, (**B**) ribo-NTPs and 3′-dATP-analog. DNA1 (1 μM) was TdT-tailed (90 min) with non-modified or base-modified nucleotides at varying NTP concentrations (1 mM, 100 μM, 10 μM, 1 μM, high to low concentration indicated by wedge). Lines indicate where different parts of same or different gels have been combined to allow comparison of samples. Phosphor imaging of radioactivity in 15% sequencing PAGE is shown. N.R.: no reaction control. Abbreviations: *E*: ethynyl, *O*: octynyl, Norb: norbornene, *PEG*N_3: azide-PEG_4-aminoallyl, *H*N_3: 6-azidohexyl, *P*: propargyl.

Staudinger ligation with DyLight 488 phosphine (2 mM), with or without the addition of helper DNA DNA9 (30 μM) at 37°C or 60°C for 2 h. Reactions with DNA9 were subjected to DNase I-digest as previously described (9).

RESULTS

Many different modified nucleotides are incorporated by TdT with varying efficiencies

To explore the substrate specificity of TdT, we screened 22 deoxy- (d), dideoxy- (dd) and (ribo)nucleoside triphosphates (NTPs) modified with clickable moieties for acceptance by the enzyme (Figure 2). To avoid complex names, abbreviations will be used throughout this article (Figure 1, Table 1). For comparison, 16 unmodified NTPs, 2′-dNTPs and ddNTPs were also tested. Under standard conditions (1 mM nucleotide, 1 μM DNA, 90 min incubation) all nucleotides were incorporated into DNA. Tail lengths and extent of substrate DNA turnover, however, varied for the different nucleotides, with turnover often reaching 100% (Figure 2). Nucleotides for labeling can thus be chosen according to the needs of each experiment. Among all tested derivatives, 5-*E*-dUTP, which structurally resembles dTTP very closely, was incorporated with the highest efficiency and longest tails (Figure 2B). All other modified 2′-dNTPs formed considerably shorter, heterogeneous tails (Figure 2B, C), and in some cases exhibited incomplete DNA substrate turnover. Interestingly, a reduction of nucleotide concentration often lead to an increase in tail lengths and turnover (Figure 3A), hinting at a substrate inhibition effect caused by the base-modified nucleotides. For base-modified analogs of ribo-NTPs (Figures 2C, D and 3B), incorporation patterns mostly resembled those of the parental ribo-NTPs, with incorporation of 1-4 nucleotides. The lowest incorporation efficiency among all analogs in this study (< 2% after 90 min) was observed for N^6-*H*N_3-ATP (Figures 2C and 3B), in which the azido moiety was attached via a long hexyl linker (for more details see Supplementary Text S1). Interestingly, the 3′-deoxy-version of this nucleotide was incorporated much more efficiently and allowed ∼100% substrate turnover at 10 μM or 100 μM nucleotide concentration, making it very attractive for efficient single labeling (Figure 3B). Finally, sugar-modified nucleotides (i.e. those carrying an azido group attached to the ribose, Figure 2E, F) showed substrate preferences sim-

Table 2. Maximum concentrations tailed efficiently with different nucleotides and conditions needed for efficient tailing

Modified nucleotide(s)	Maximum concentration and conditions for efficient tailing
2′-N_3-C/GTP, 5-*PEG*N_3-dUTP, N^6-*H*N_3-3′-dATP, 5-*E*-UTP, 5-*O*-dC/UTP, N^6-*P*-ATP, DIBAC-dUTP, Norb-dCTP	100 μM - 90 min
8-N_3-ATP	100 μM – overnight
3′-N_3-ddCTP	100 μM – overnight; 50 μM - 90 min
5-*E*-dUTP	50 μM – 90 min (quantitative)
2′-N_3-UTP	50 μM – 90 min (near-quantitative)
2′-N_3-ATP	50 μM – overnight (near-quantitative)
3′-N_3-ddGTP	25 μM – 90 min (near-quantitative)
3′-N_3-ddTTP	5 μM – 90 min (∼80%); 5 μM overnight (> 85%)
3′-N_3-ddATP	5 μM – overnight (∼50%)

DNA1 or DNA4 and 1 mM NTP concentrations were employed, except for N^6-*H*N_3-3′-dATP (200 μM).

ilar to non-modified ribo-nucleotides (G most and A least preferred) but were incorporated less efficiently than the respective non-modified ribo- or ddNTPs (see also Supplementary Figure S1). Here, contrary to some base-modified analogs, a reduction of nucleotide concentration did not increase incorporation efficiency (Supplementary Figure S2).

Taken together, for many of the tested nucleotides efficient tailing reactions can be performed, so that these nucleotides can be employed for chemo-enzymatic labeling of DNA. Users can choose between different modified nucleotides to tail DNA with many (5-*E*-dUTP) or a few modified nucleotides, where more heterogeneous tails are formed with most base-modified 2′-dNTPs and 2′-N_3-GTP, and more defined ones (1-4 nt) with most base-modified ribo-NTPs or 2′-N_3-NTPs. It is equally possible to add a single modified nucleotide, using either 3′-N_3-ddNTPs or 3′-*H*N_3-3′-dATP. For a selection of efficiently incorporated nucleotides, the dynamic range of TdT-catalyzed incorporation was tested by increasing DNA concentrations up to 100 μM (Table 2). Even at such high concentrations, with many of them, DNA was tailed with quantitative turnover after only 90 min reaction, so these nucleotides can be used to modify highly concentrated DNA.

TdT tailing depends on DNA structure

The structure and strandedness of DNA tailing substrates has been shown previously to influence the outcome of the tailing reaction. Co^{2+}, which was present in TdT buffers used throughout this study, is known as an additive that improves tailing efficiencies for dsDNA (39). Comparing tailing of ds and ssDNA for a selection of modified NTPs, incorporation patterns were similar in most cases, but several modified NTPs (Supplementary Figure S3) showed decreased TdT tailing yields for dsDNA, though not less than ∼40%. Near-quantitative turnovers were achieved with 2′-N_3-UTP and 5-*E*-UTP. To preserve high efficiencies for labeling of dsDNA, we thus recommend either NTPs that are incorporated fast, such as 5-*E*-UTP or 2′-N_3-UTP or to extend the incubation time. Taking this into consideration, it will be possible to tail DNA of any origin, be it chemically or enzymatically synthesized, or isolated from natural sources, similar to what we have shown for RNA (9) (see Supplementary Text S2 for a more detailed comparison of RNA and DNA tailing).

Click reactions proceed efficiently on TdT-tailed DNA

After incorporation of the modified nucleotides into the DNA in a first step, in order to attach the label of choice, the respective click reaction is performed in a second step (Figure 1A). For each of the four functional groups (alkyne, azide, DIBAC, norbornene) we tailed DNA with at least one modified nucleotide bearing that group. We then subjected the tailed DNA to all click reactions possible with those functional groups, attaching different fluorophores (Alexa Fluor 647 – AF647, Cy5, or DyLight 488) or biotin (Figures 4 and 5, Supplementary Figures S4–7). CuAAC of alkyne-tailed DNA with different azides generally proceeded with near-quantitative yields (Figure 4A–C). All tailing product bands disappeared, while the appropriate number of CuAAC-product bands with lower electrophoretic mobility appeared. Only for DNA tailed with 5-*E*-UTP (alkyne directly attached to the base), CuAAC yields were visibly below 100%, presumably either due to greater sterical hindrance (26) or partial hydration of the base-linked alkyne (45). Similarly, SPAAC and DAR_{inv} proceeded quantitatively on DNA tailed with 5-DIBAC-dUTP (10 μM) or norbornene-bearing dNTPs (Figure 4C, D). For the norbornene-bearing dNTPs major TdT-tailing products were bearing one to seven modifications. All tailing product bands disappeared after DAR_{inv}, while bands for single and double Cy5-modified DNA appeared (Figure 4D). DNA bearing more than two Cy5-modifications after DAR_{inv} seemed to remain in the gel pockets (Figure 4D), so that DAR_{inv} yield was not quantified for species bearing more than two norbornene modifications, but is expected to be quantitative due to the complete disappearance of tailed DNA.

Of interest to prospective users, it was possible to shorten the workflow by avoiding the purification step after TdT tailing when low nucleotide concentrations (10 μM) were used, as exemplified for DNA tailed with 5-*O*-dUTP, 5-DIBAC-dUTP or Norb-dNTPs (Figure 4C, D), thus avoiding laborious purification and material losses.

The attachment of N_3-nucleotides enables the conversion of DNA by CuAAC, SPAAC and Staudinger ligation. As shown above, we were able to incorporate five different classes of N_3-bearing nucleotide analogs (Figure 5A), in which the N_3-moiety was attached to the nucleotide at different positions (C-2′, C-3′, C-5, N^6, C-8), either directly or via different linkers (hexyl- or PEG-linker). This prompted

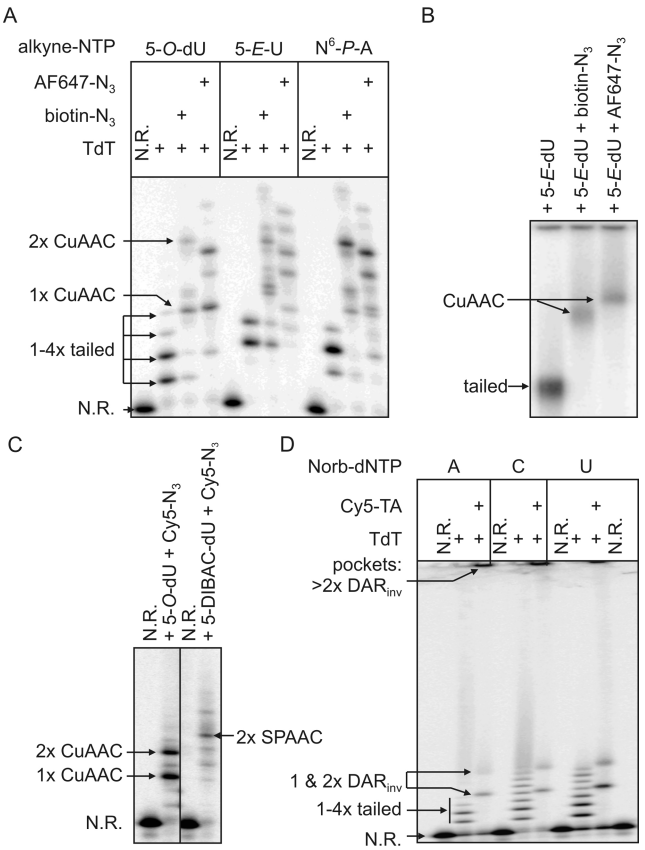

Figure 4. Click reactions of DNA2, after TdT reaction with 5-*E*-UTP, 5-*O*-dUTP or N^6-*P*-ATP (**A**), 5-*E*-dUTP (**B**), 5-*O*-dUTP or 5-DIBAC-dUTP (**C**) or with different Norb-dNTPs (**D**). CuAAC was carried out after precipitation of TdT products (A ,B) or without precipitation (C); SPAAC (C) or DARinv (D) without purification of TdT products. In all cases, click reactions lead to clear band shifts. In (D) sequences carrying >2 modifications disappear after DAR_{inv}, while a strong DNA signal appears in the pocket. Radioactive signals in 15% sequencing PAGE are shown. See Supplementary Figure S4 for additional fluorescent scans and overlays of both, which were used to confirm the identity of products. N.R.: no reaction control/not reacted. Abbreviations: *E*: ethynyl, *O*: octynyl, Norb: norbornene, *P*: propargyl.

us to ask whether the chemical environment influences azide reactivity.

Click reactivity depends on azide attachment

To address this question, DNA was tailed with 2′-N_3-ATP, 3′-N_3-ddATP, N^6-*H*N_3-3′-dATP, 8-N_3-ATP or 5-*PEG*N_3-dUTP, under conditions that led to > 40% single incorporation. Tailed DNA was then reacted in any of the three reactions, at varying concentrations of the respective reaction partners (2.5 μM to 2 mM; see Supplementary Figures S5–7 for examples). Yields are shown in Figure 5B and the concentrations of reaction partners needed to achieve yields ≥ 80% are summarized in Table 3.

As may be expected due to the similarity of both reactions, CuAAC and SPAAC revealed similar substrate preferences: 8-N_3-A≪3′-N_3-ddA<5-*PEG*N_3-dU<2′-N_3-A<N^6-*H*N_3-3′-dA (Figure 5B). Under the chosen conditions, yields ≥80% were achieved for all modifications, except 8-N_3 in both cycloadditions (Table 3). For the latter, yields did not exceed ∼50–60%, in line with previous observations that aryl azides are less reactive than alkyl azides in CuAAC (30,46). While efficient labeling by CuAAC or SPAAC can thus be achieved with almost all azido-nucleotides studied here, N^6-*H*N_3-3′-dATP should be favored for highest efficiency in single labeling.

A very different order of preference was observed for the Staudinger ligation: N^6-*H*N_3-3′-dA≪5-*PEG*N_3-dU<3′-N_3-ddA≪2′-N_3-A<8-N_3-A. Here, contrary to CuAAC and SPAAC, yields > 80% were achievable for DNA containing 8-N_3-A and 2′-N_3-A, but none of the other modifications under the chosen conditions. 8-N_3-A's high reactivity towards phosphines, even after prolonged incubation at 37°C, also demonstrates that the azide is fairly stable in this analog, unlike previously observed for 5-N_3-dU, another nucleotide analog bearing a base-linked azide (46). Our observations correlate with other reports of the aryl azide of 8-N_3-A reacting much more readily than an alkyl-linked azide (30) and PEG-linked azide being more reactive than an azide linked via a 5-carbon-atom linker (47) in the Staudinger ligation. Strikingly, for this reaction, the order of reactivity follows the predicted strength of inductive or resonance effects (caused by neighboring heteroaromatic rings or heteroatoms; Figure 5A). This may affect azide polarization and lead to stabilization of intermediates (Figure 5C), as previously described for phenyl azide (48). Importantly, although the aza-ylide intermediate was formed very fast with phenyl azide, the Staudinger ligation reaction was completed very slowly due to the stabilization of the intermediate (48). As our assay does not distinguish between intermediate and end product, this may be true for 8-N_3-A too, and may influence its usability. While the simple attachment of a functional group should be achieved readily by the irreversible formation of the aza-ylide form, experiments using pre-fluorophores (49) that require the Staudinger ligation to be completed before they fluoresce may be hampered, if instant light-up is desired.

Taken together, the choice of attachment position, linker and the type of reaction for any given experiment are critical when labeling biomolecules via click chemistry, a fact that seems to be underestimated by many researchers. Although the label to be attached, and the reaction partner (e.g. DIBAC instead of other strained alkynes) used in the chemical reaction might equally influence the reactivity, we assume the orders of preference to be similar with other labels/reaction partners. Noteworthy, the 2′-N_3, which has been the modification primarily studied in our earlier work on RNA labeling (9), is the most versatile modification for DNA labeling, since it is highly reactive in all three click reactions, in contrast to hexyl-N_3, which performs well only in CuAAC and SPAAC, or 8-N_3-A, which reacts readily only in Staudinger ligation. This finding prompted us to apply Staudinger ligation conditions similar to those used here to our recently published 3′-terminal and internal RNA labeling protocol (9), and, as expected, we obtained excellent labeling efficiencies (Supplementary Figure S8).

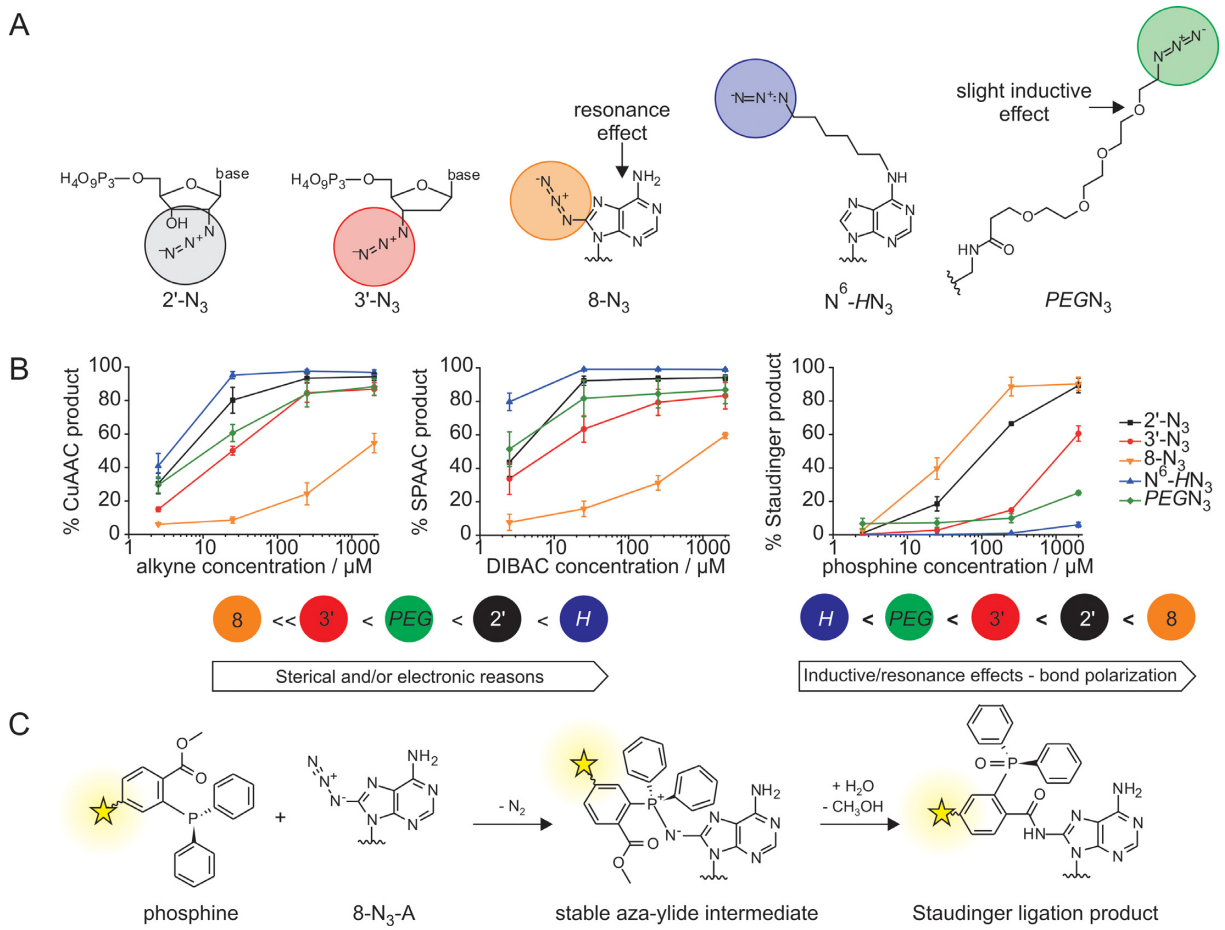

Figure 5. Influence of attachment position and linker on reactivity of azides in tailed DNA in CuAAC, SPAAC and Staudinger ligation. (**A**) Overview of azido-nucleotides incorporated. (**B**) Reaction yields in the different click reactions, as determined by 15% sequencing PAGE. Data points represent mean ± S.D. of yields measured for a defined sequence (DNA2) and a randomized sequence (DNA3) (single measurement for each). Note that the assessment of yield was potentially biased for *PEG*N_3, due to blurred bands. Substrate preference in the cycloadditions (CuAAC, SPAAC) and in the Staudinger ligation is indicated. In Staudinger ligation, reactivity is proportional to azide-bond polarization as predicted by expected inductive effect of neighboring groups, which, among the aliphatic azides, should be highest for 2′-N_3 (proximity of the nucleobase, neighboring 3′-hydroxyl group), followed by 3′-N_3, and PEG-linked azide, where the ether bond may still cause a slight inductive effect. (**C**) Stabilization of aza-ylide intermediate in the reaction between DyLight 488 phosphine and 8-N_3-A (adapted from (48)).

Table 3. Reactant concentrations needed for yields ≥ 80%

Reaction	Azide	Reactant-concentration
CuAAC	hexyl-N_3	25 µM
	PEGN_3	250 µM
	2′-N_3	25 µM
	3′-N_3	250 µM
	8-N_3	not possible (~50% at 2 mM)
SPAAC	hexyl-N_3	2.5 µM
	PEGN_3	25 µM
	2′-N_3	25 µM
	3′-N_3	250 µM
	8-N_3	not possible (~60% at 2 mM)
Staudinger ligation	hexyl-N_3	not possible (~5% at 2 mM)
	PEGN_3	not possible (~25% at 2 mM)
	2′-N_3	2 mM
	3′-N_3	not possible (~65% at 2 mM)
	8-N_3	250 µM

Reaction conditions were the following. CuAAC: 100 µM $CuSO_4$, 500 µM THPTA, 1 mM sodium ascorbate, 2 h, 37°C; SPAAC: 20 min, 65°C, Staudinger ligation: 2 h, 60°C. Similar trends were observed for SPAAC under different conditions (2 h, 37°C).

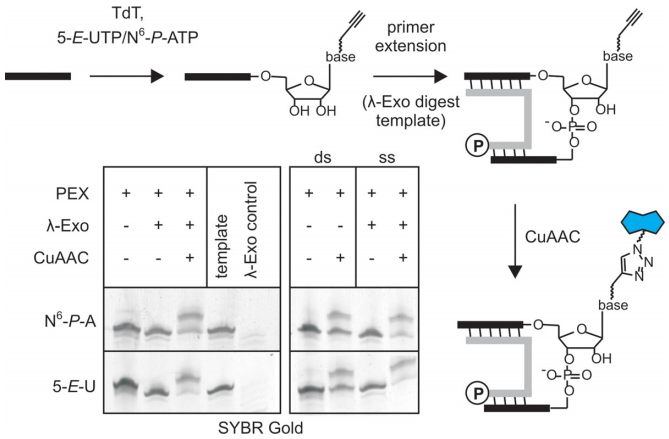

Figure 6. Internal modification of DNA. DNA is first tailed with either 5-*E*-UTP or N^6-*P*-ATP, and then elongated by primer extension. The 5′-monophosphorylated template (shown in gray) is optionally digested with λ-exonuclease (λ-Exo) and the alkyne is reacted in CuAAC, to attach biotin to the single-stranded (ss) or double-stranded (ds) DNA. 12% denaturing PAGE, visualization by SYBR Gold staining.

Internal DNA labeling

Inspired by our success in converting 3′-terminal modifications of RNA into internal ones by tailing or ligating the 3′-terminally azido-modified RNA, we asked whether this would be possible for DNA labeling as well (note that internal labeling here should not be confounded with click ligation (20,50–53), see Supplementary Figure S9). As discussed in Supplementary Text S2, it was not possible to achieve as markedly a preference for single incorporation of modified 2′-dNTPs or ribo-NTPs as in our RNA labeling experiments. On the other hand, 3′-blocked nucleotides would not allow for further manipulation of the 3′-terminus. However, several nucleotides exhibited reasonable preferences for single incorporation, especially when low NTP-excess was used. These include the ribo-nucleotides 5-*E*-UTP and N^6-*P*-ATP (Figure 3B). We thus prepared primers that were tailed with (mostly a single) 5-*E*-U or N^6-*P*-A moiety, and either performed primer extension (PEX; Figure 6) or splinted ligation (Supplementary Figure S10), using reverse complementary, 5′-phosphorylated DNA templates, which we removed in some cases, using λ-exonuclease. We then subjected the products to CuAAC with biotin-N_3 and detected shifted products by PAGE (Figure 6). Due to the fact that < 100% of primers indeed contained the alkyne, and possibly due to incomplete CuAAC conversion, we estimate the efficiency to be ∼80% in case of 5-*E*-U-labeled ssDNA and ∼65% in case of N^6-*P*-A-labeled ssDNA, according to non-specific DNA staining. CuAAC-conversion of dsDNA was equally possible and rather efficient (Figure 6), which is interesting with respect to labeling, e.g. of PCR products.

DISCUSSION

In our current work, we show that TdT can be employed to incorporate a diverse range of modified nucleotides that contain ‘clickable’ moieties, namely alkynes, azides, DIBAC or norbornene attached at various positions. We made use of these moieties to successfully couple DNA to various fluorophores and biotin, attaching a single or several labels of interest. With its high dynamic range (tested on up to 100 μM DNA concentrations) and successful application to dsDNA, this method provides the possibility to enzymatically label both, high and low amounts of DNA, in ss or ds conformation. Although demonstrated by us on short DNAs for ease and precision of analyses, our methods will be of particular interest for labeling of longer DNAs, e.g. to post-synthetic labeling of PCR products, plasmid- or genomic DNA, which are not easy to label with most existing methods. Beyond the attachment of labels to any kind of DNA, further applications include click-ligation (20,50–53) and construction of conjugates with surfaces (54,55) and nanoparticles (56). Having shown previously that DAR_{inv} and CuAAC can be applied simultaneously, in an orthogonal fashion, and our new method being specific to the 3′-terminus, our approach can further be interfaced with methods that introduce labels at the 5′-terminus or at internal positions, e.g., using labeled primers, to achieve multiple labeling [manuscript in preparation]. This could be exploited for instance to attach strands to the tips and surfaces used in atomic force microscopy (57) or to produce molecular beacons (31).

With our work, we also provide the first systematic study about the influence of the chemical environment on azide-reactivity in the context of biomolecule labeling. Interestingly, azides linked to flexible linkers, which might be expected to be sterically least hindered and therefore most reactive, do not always react very readily. Instead, the reactivity of each azide depends on the reaction in which it is used. Therefore, great care should be taken by researchers who use click reactions to label biomolecules. Of interest, the 2′-azide, which we had chosen for our recently published RNA labeling approach, turned out to be the most versatile modification in our settings, as it reacted very efficiently in all three click reactions tested (CuAAC, SPAAC and Staudinger ligation).

In addition to providing a highly modular and efficient method for 3′-functionalization, in which the choice of labels is virtually unlimited, we also show that, due to the presence of free 3′-OH-functionalities in many of our modified nucleotides, it is possible to convert the 3′-terminal modification into a site-specific internal one, similar to our previous reports for RNA (9). This makes DNA amenable not only to internal attachment of different labels, but also to branching and crosslinking (35,58,59).

Taking into account that most of the nucleotides and modification reagents shown here are commercially available and that we can achieve site-specific internal labeling or 3′-terminal labeling, with long or short homopolymeric tracts, as well as single nucleotides, we believe that our new labeling strategy will be of great use to a wide range of researchers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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