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REVIEW

Cycloadditions for Studying Nucleic Acids

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Abstract Cycloaddition reactions for site-specific or global modification of nucleic acids have enabled the preparation of a plethora of previously inaccessible DNA and RNA constructs for structural and functional studies on naturally occurring nucleic acids, the assembly of nucleic acid nanostructures, therapeutic applications, and recently, the development of novel aptamers. In this chapter, recent progress in nucleic acid functionalization via a range of different cycloaddition (click) chemistries is presented. At first, cycloaddition/click chemistries already used for modifying nucleic acids are summarized, ranging from the well-established copper(I)-catalyzed alkyne–azide cycloaddition reaction to copper free methods, such as the strain-promoted azide–alkyne cycloaddition, tetrazole-based photoclick chemistry and the inverse electron demand Diels–Alder cycloaddition reaction between strained alkenes and tetrazine derivatives. The subsequent sections contain selected applications of nucleic acid functionalization via click chemistry; in particular, site-specific enzymatic labeling in vitro, either via DNA and RNA recognizing enzymes or by introducing unnatural base pairs modified for click reactions. Further sections report recent progress in metabolic labeling and fluorescent detection of DNA and RNA synthesis in vivo, click nucleic acid ligation, click chemistry in nanostructure assembly and click-SELEX as a novel method for the selection of aptamers.

Keywords Nucleic acid · Cycloaddition · Click chemistry · CuAAC · iEDDA · Metabolic labeling

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1 Introduction

This chapter gives an overview about applications of cycloaddition reactions to study nucleic acids. These cycloaddition reactions are typically grouped under the heading "click chemistry" despite varying other chemistries, and an enormous amount of different applications in the context of DNA and RNA have been found so far. Only selected recent implementations for cycloaddition reactions on nucleic acids can be presented in the limited scope of this chapter. Today's click reactions, as first defined by Sharpless et al. [1], are associated with biocompatibility, fast reaction kinetics and bio-orthogonality.

Applications of cycloaddition reactions on nucleic acids span from global or site-specific labeling of modified oligonucleotides in vitro for structural and functional studies on nucleic acids, towards in vivo applications of click reactions such as metabolic DNA and RNA labeling, modification of oligonucleotides for antiviral and anti-tumor therapies and the regulation of gene expression. Moreover, cycloaddition click chemistry has been used to build up DNA and RNA strands with artificial backbones and for DNA nanostructure assembly. Examples for these applications will be given in the following sections.

All three main regions of nucleic acids have been targeted for chemical modifications that can undergo cycloaddition reactions: the nucleobases, the (deoxy-)ribose moiety and the phosphodiester backbone. The most widespread type of modifications are nucleobase alterations for the introduction of novel base analogues, bioconjugation or attaching fluorophores for structural and functional studies.

Chemically modified nucleosides can be introduced into nucleic acid by either synthetic methods during solid phase oligonucleotide synthesis or via enzymatic reactions. The site-specific functionalization of nucleic acids proceeds primarily via solid phase DNA/RNA synthesis. Artificial nucleosides that possess reactive groups for click chemistry (at the nucleobase or at sugar moiety) are introduced as phosphoramidites, either internally or at the 5′-end of the synthesized oligonucleotide. Moreover, the use of solid supports bearing modified nucleobases allows the introduction of 3′ modifications. These methods are widely employed for the site-specific attachment of labels such as fluorogenic tags, spin-labels or biotin to oligonucleotides, and are highly relevant for structural and functional studies of these nucleic acids [2, 3]. Requirements for solid-phase oligonucleotide synthesis using modified nucleobases are, first of all, the compatibility of the introduced functional group with the reaction conditions of solid-phase synthesis. Moreover, limitations in length do exist for solid-phase DNA and RNA synthesis (shorter than 100 nucleotides for RNA synthesis); these restrict investigation of site-specifically modified long oligonucleotides such as long non-coding RNA molecules with a length of several hundred nucleotides.

A different approach is the high-density functionalization of DNA and RNA via enzymatic methods. Here, modified nucleobases are introduced in vitro into oligonucleotides via enzymatic reactions, namely PCR and in vitro transcription. Various polymerases accept small-sized modifications on nucleobases, such as

alkyne moieties, if the formation of Watson–Crick hydrogen bonds is not hampered by the modification [4–10]. Furthermore, end-labeling of enzymatically prepared RNA molecules can be achieved using the corresponding for post-synthetic click chemistry modified starter nucleotides for T7 in vitro transcription. For a recent example, see Jäschke et al. [11]. Here, RNA labeling was achieved using an alkyne-modified dinucleotide as starter nucleotide for in vitro transcription. The 5′-end of the transcript remains unaltered, allowing ligation to other RNA molecules and resulting in an internally modified RNA oligonucleotide [11].

In the first section of this chapter, the different cycloaddition/click chemistries already used for modifying nucleic acids are summarized. The subsequent sections contain selected applications and themes where cycloaddition reactions are a central element of studies on nucleic acids.

2 Cycloaddition Reactions on Nucleic Acids

The following graphic (Fig. 1) gives an overview about various cycloaddition reactions used for nucleic acid functionalization. The individual reactions are discussed in detail below.

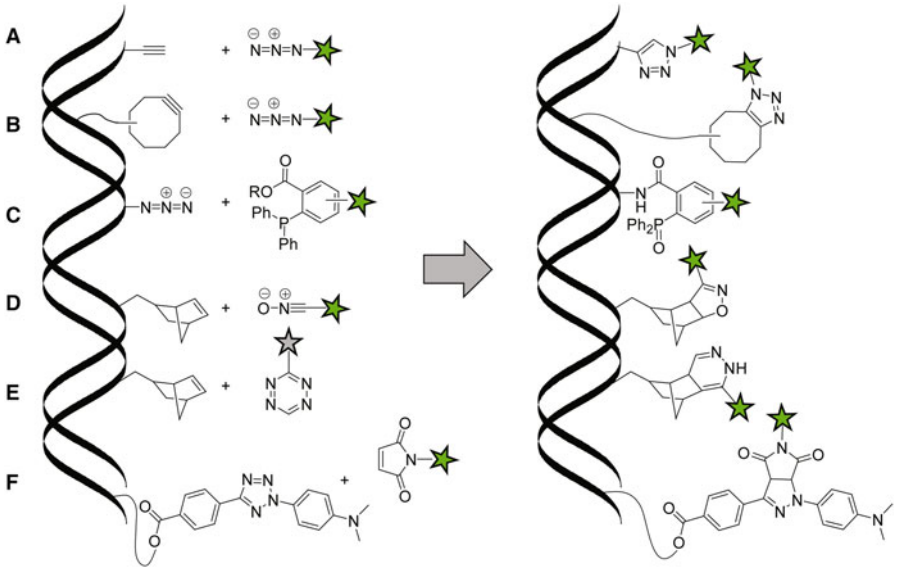

Fig. 1 Cycloaddition reactions employed in nucleic acid labeling with reporter groups (*green star*). *A* Cu^{I}-mediated azide–alkyne cycloaddition (CuAAC) of a terminal alkyne with an azide. *B* Strain-promoted azide–alkyne cycloaddition (SPAAC) of an azide with a cyclooctyne derivative. *C* Staudinger ligation of an azide with a phosphine derivative (not a cycloaddition reaction, see below). *D* Norbornene cycloaddition of a nitrile oxide as 1,3-dipole and a norbornene as dipolarophile. *E* Inverse electron-demand Diels–Alder cycloaddition reaction between a strained double bond (norbornene) and a tetrazine derivative. *F* Photo-click reaction of a push–pull-substituted diaryltetrazole with an activated double bond (maleimide)

2.1 Copper(I)-Catalyzed Alkyne–Azide Cycloaddition (CuAAC) on Nucleic Acids

The copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC) reaction [1] (Fig. 1A) has been extensively employed for the site-specific labeling of oligonucleotides with various reporter groups [3, 4, 7, 9, 12–16], ligating DNA strands [17, 18], cross-linking complementary strands [19], for surface functionalization [20] and for the formation of bimetallic Ag–Au nanowires from DNA templates [21]. Selected examples are described in the following section.

Alkyne moieties can easily be incorporated into DNA and RNA strands during solid phase synthesis and the site-specific incorporation of multiple, even consecutive labels into DNA has been demonstrated [12]. Using appropriate copper(I)-complexing ligands [22] to prevent copper-catalyzed cleavage of DNA [23], azide-modified sugars and fluorophores react efficiently with alkyne-modified DNA [12]. The site-specific incorporation of different reporter groups, which is, for example, desired to study nucleic acid folding via Förster resonance energy transfer (FRET) using an appropriate FRET pair as fluorophores, can also be achieved by postsynthetic click modification. For this, alkyne functionalized nucleobases possessing orthogonal protecting groups (TMS, TIPS) are introduced as phosphoramidites during solid phase DNA synthesis [13]. Selective stepwise deprotection of alkyne moieties allows labeling of DNA oligonucleotides with up to three different reporter groups [13].

CuAAC click chemistry has further been used to cross-link complementary DNA strands to form stable duplexes employing alkyne- and azide-modified nucleobases. Increased melting temperatures up to 30 °C could be achieved [19]. PNA/DNA-peptide conjugates can easily be prepared by CuAAC click chemistry using 3′-azido-modified oligonucleotides and alkyne-modified peptides [24, 25].

Furthermore, copper catalyzed click chemistry allowed the assembly of catalytically active ribozymes with RNA strand lengths beyond the scope of solid phase RNA synthesis. In this context, the hairpin ribozyme was reconstructed from chemically synthesized RNA strands, which were covalently cross-linked by a CuAAC reaction [26]. To prevent RNA degradation, Cu^{II} sulfate and sodium ascorbate for in situ generation of the catalyst and a Cu^{I} stabilizing ligand are usually employed [22]. Azide functionalities were introduced into RNA strands via a two-step method by postsynthetically reacting amino-modified uridines with N-hydroxysuccinimide esters of an azide-modified carboxylic acid [26]. For the hammerhead ribozyme, an intrastrand click-ligation of RNA strands bearing 3′-alkyne and 5′-azide modifications gave rise to active ribozyme constructs with an artificial triazole backbone at the active site [26].

Nitroxide radical spin labels can be used to measure inter-spin distances by pulsed electron paramagnetic resonance (EPR) spectroscopy, and to allow study of structure and folding of DNA and RNA oligonucleotides [27–30]. Spin-labeling of nucleobases by CuAAC in solution has first been demonstrated using the radical azide 4-azido-2,2,6,6-tetramethylpiperidine 1-oxyl (4-azido-TEMPO) [31]. Alkyne modifications at position 7 of 7-deazapurines and C5-modified pyrimidines allowed attachment of the spin label in the major groove of duplex DNA [31]. In the same year, click chemistry on solid support to introduce spin labels into DNA was reported [32].

The incorporation of azides into DNA and RNA during solid phase synthesis has long been neglected due to incompatibility of phosphoramidite chemistry with azido groups because of Staudinger-type side-reactions [33, 34]. Instead, enzymatic methods were employed to incorporate azido-modified nucleobases as triphosphates into DNA [6, 35]. Furthermore, H-phosphonate chemistry allowed the intrastrand introduction of azide labels into short DNA oligonucleotides synthesized on solid support [36]. In 2011, Micura et al. demonstrated efficient preparation of C2′ azido-modified RNA by solid phase RNA synthesis using novel azido-modified nucleoside phosphodiester building blocks [37, 38]. All four 2′-azido–modified canonical nucleobases, 2′-azido-2′-deoxyuridine [37], 2′-azido-2′-deoxyadenosine [37], 2′-azido cytidine [38] and 2′-azido guanosine [38] were used in solid phase RNA synthesis and postsynthetic fluorescent labeling via CuAAC click chemistry using alkyne-modified fluorophores. Additionally, 2′-azido groups were well tolerated in the guide strand of siRNAs, demonstrated by siRNA induced silencing of the brain acid soluble protein 1 (BASP1) encoding gene in chicken fibroblasts [38]. In 2014, preparation of a 2′-azido–modified solid support for automated RNA synthesis compatible with phosphoramidite chemistry for 3′-terminal labeling of oligoribonucleotides was reported [39].

2.2 Copper-Free Click Chemistry for Nucleic Acid Functionalization

The presence of Cu^{1} ions leads to RNA damage and is toxic for in vivo systems. Copper-free click reactions are therefore highly desirable. Several groups established copper-free cycloaddition reactions on nucleic acids. Recent developments in the field are presented in the following sections in detail. In the same context, a modified Staudinger reaction has been employed for bioorthogonal labeling of nucleic acids; for completeness, this method is therefore also referenced briefly below.

2.2.1 Staudinger Ligation

The Staudinger ligation, adapted by Bertozzi et al. for orthogonal labeling of biomolecules [40] yields a stable amide bond by reaction of an azido-modified molecule and a triarylphosphine derivative (Fig. 1C). Incorporation of azides into nucleic acids is a prerequisite for application of this bioorthogonal labeling reaction, which is problematic during solid phase synthesis as discussed above. However, the Staudinger ligation reaction has successfully been used for DNA labeling with fluorescent reporter groups employing a 5′-azido–modified oligonucleotide [41]. Enzymatic incorporation of azido-modified 2′-deoxyuridine and 2′-deoxyadenosine triphosphates during PCR reactions allowed internal labeling of DNA strands via Staudinger ligation with biotin or fluorophore-modified phosphines [6, 42, 43]. Due to the complex synthesis of azido-modified nucleic acids, combined with the poor stability of phosphines in solution and relatively slow reaction kinetics, focus shifted more and more towards the development of other catalyst-free bioorthogonal reactions, such as the strain-promoted azide–alkyne cycloaddition discussed in the following section.

2.2.2 Strain-Promoted Azide–Alkyne Cycloadditions

A particular major limitation of the CuAAC reaction is metal-catalyzed strand degradation [22, 23, 44], which has been partially overcome by the development of novel Cu^1 stabilizing ligands [45–47]. However, the cell toxicity of copper complexes remains challenging for live cell and in vivo applications. The strain-promoted azide–alkyne cycloaddition (SPAAC, Fig. 1B) between azides and strained cyclooctynes [48, 49] such as dibenzocyclooctyne allows bioorthogonal reactions on nucleic acids in the absence of copper. Solid phase RNA synthesis using a strained cyclooctyne phosphoramidite for 5′-end functionalization and reaction with azido-modified peptides and oligosaccharides [50] and 5′-end cyclooctyne modification of RNA during solid phase synthesis [51], non-nucleoside alkyne monomers for RNA labeling [52], as well as template directed DNA strand ligation [53] and internal and terminal DNA labeling with dibenzocyclooctyne [54, 55], were reported. 5′-end modified DNA with an achiral bicyclo [6.1.0] nonyne phosphoramidite during solid phase DNA synthesis can also be used for SPAAC yielding only enantiomeric products [56]. Conjugation to lipophilic polymers has been demonstrated [56].

SPAAC has further been employed for RNA labeling with 2′-azido-modified nucleotides incorporated at the 3′-end of RNA oligonucleotides by an enzymatic approach with poly(A) polymerase [7]. Ligation of these 3′-azido modified RNAs employing a splinted ligation approach allows the preparation of internally azido-modified RNAs [7]. Besides this, azido-modified capped RNA was successfully labeled via SPAAC [14].

Gold nanoparticles coated with single stranded DNA bearing a 3′-azide or 5′-alkyne modification were covalently linked together using a splint DNA strand to promote the SPAAC reaction demonstrating applications of SPAAC reactions in programmed nanoparticle organization [57].

Bibenzocyclooctyne and bicyclo [6.1.0] non-4-yne-modified 2′-deoxyuridine triphosphates can be further employed for PCR reactions, with standard DNA polymerases allowing internal labeling of DNA with multiple strained alkyne residues [10]. Fluorescent labeling was demonstrated with azide-modified fluorophores [10].

In summary, SPAAC reactions with dibenzocyclooctyne-modified nucleic acids have proven to be valuable tools for a catalyst-free bioorthogonal click reaction with biologically inert, non-natural azides. One drawback is the rather slow reaction with second order rate constants of approximately 0.05 M^{-1} s^{-1} [50, 58]. Wagenknecht et al. [59] developed a carboxymethylmonobenzocyclooctyne-modified 2′-deoxyuridine phosphoramidite which possesses increased reactivity towards azide-modified reporter groups ($k_2 = 0.8$ M^{-1} s^{-1} in aqueous solution). Incorporation into DNA during solid phase DNA synthesis allowed postsynthetic SPAAC reaction with an quinolinium-styryl-coumarin azide dye probe. Click reaction results in a strong increase in fluorescence intensity of the dye [59].

2.2.3 1,3 Dipolar Cycloadditions with Nitrile Oxides (Nitrile Oxide–Norbornene Click Chemistry)

Nitrile oxides react with strained alkenes in a Huisgen 1,3 dipolar cycloaddition reaction (Fig. 1D). This bioorthogonal reaction has been employed for labeling of DNA during automated solid phase synthesis. Nitrile oxides are strong electrophiles; however, the cross reaction with natural nucleobases is rather slow compared to the reaction with the norbornene double bond [60]. Furthermore, the reaction of styrene-modified DNA with nitrile oxides was demonstrated, yielding only one regioisomer, 3,5-disubstituted isoxazoline [5]. Styrene functionalized nucleobases were either incorporated during solid phase DNA synthesis or as triphosphates in PCR reactions of fragments up to 900 base pairs. The CuAAC click chemistry is orthogonal to the 1,3 dipolar cycloaddition, with nitrile oxides allowing high density functionalization of DNA with two different reporter groups [5].

2.2.4 Photo-Click Reaction on Nucleic Acids

Recently, tetrazole-based photoclick chemistry, which has previously been used for protein labeling in live cells [61], has been applied for postsynthetic DNA modification [62]. 2′-Deoxyuridine was modified with a substituted diaryltetrazole, bearing an electron-donating dimethylamino group at one end and an electron-withdrawing carboxyl group at the other end, and incorporated into DNA as phosphoramidite building block during solid phase DNA synthesis (Fig. 1F). Postsynthetic labeling is feasible using maleimide-modified fluorophores as dipolarophiles and irradiation at a wavelength of 365 nm [62]. The rate constant of the photo-click reaction is in the range of other fast copper-free bioorthogonal reactions such as Diels–Alder cycloaddition reactions. An advantage of this photo-click reaction is the fact that the reaction only proceeds upon irradiation. This allows an exact timing of the reaction, which might be of interest for spatiotemporal control in live cell applications [62].

2.2.5 Inverse Electron Demand Diels–Alder Cycloaddition (iEDDA) Reaction

The inverse electron demand Diels–Alder cycloaddition (iEDDA) reaction between strained alkenes and tetrazine derivatives has gained more and more attention for orthogonal labeling of biomolecules in the last years (Fig. 1E) [63]. This catalyst-free reaction can be extremely rapid in the case of *trans*-cyclooctenes as reactants with second order rate constants of up to 380,000 M^{-1} s^{-1} in aqueous solutions [64], and reactions on genetically encoded alkenes in proteins and on alkene functionalized small molecules can be performed in living cells [65–70]. The reaction is highly specific and cell toxicity of the tetrazine derivatives is negligible [66, 71, 72]. Moreover, tetrazine–fluorophore conjugates possess strong turn-on fluorescence after reaction with dienophiles that strongly depends on the fluorophore employed (fluorophore emission between 510 and 570 nm) [73–75]. This is an extremely important feature for labeling procedures in living cells and in vivo, where removal of unreacted probes is virtually impossible.

To date, a few examples of iEDDA reactions on nucleic acids in vitro and in cells were reported. In 2010, Jäschke et al. [76] reported the first example of DNA modification by the inverse-electron-demand Diels–Alder reaction between norbornene dienophiles and tetrazine-derivatives. 3′- and 5′-terminal labeling, as well as internal modification of DNA with norbornenes and subsequent iEDDA reaction, was demonstrated [76]. Yields of 96 % are observed using a 1:1 stoichiometry of DNA and tetrazine derivative [76]. Lower reactant concentrations and considerably lower excess of labeling reagent (usually 1:3 stoichiometry) compared to CuAAC, as well as the absence of any toxic catalysts, shows the potential of this cycloaddition reaction for in cell and in vivo applications.

CuAAC and iEDDA reactions are fully orthogonal and can be employed for dual labeling strategies of DNA in a one-pot reaction [64]. Facile preparation of dual labeled FRET probes for biophysical studies on nucleic acids is possible by this approach [64].

The iEDDA reaction was further employed for template-directed ligation of DNA using fluorescent DNA probes consisting of quenched tetrazine–fluorophore conjugates and methylcyclopropene groups as dienophiles [77]. This method could in principle allow fluorescent detection of specific DNA and RNA sequences in living cells.

Besides stable norbornene and slightly more reactive methylcyclopropene dienophiles, *trans*-cyclooctenes have also been incorporated into DNA during PCR as *trans*-cyclooctene–modified thymidine triphosphate and reacted with tetrazine derivatives in an iEDDA reaction [78].

The iEDDA reaction is also compatible with RNA. 5′-End labeling of an RNA modified with a 5′-norbornene phosphoramidite during solid phase RNA synthesis was performed [79]. In 2014, we demonstrated intra-strand labeling of norbornene-modified RNA oligonucleotides by iEDDA in vitro and in a cellular context [80]. For this, a norbornene-modified uridine phosphoramidite was introduced into RNA during solid phase RNA synthesis. Transfection of norbornene containing siRNA duplexes into mammalian cells and subsequent reaction with tetrazine–fluorophore derivatives in the cytoplasm of cells proved that the iEDDA reaction can in principle be used to label nucleic acids in living cells [80].

RNA-peptide conjugates have also been prepared by the inverse electron demand Diels–Alder reaction using tetrazine-modified peptides and enzymatically 5′-dienophile–modified RNA [81]. Moreover, enzymatic RNA labeling using *trans*-cyclooctene–modified cytidine triphosphate during in vitro transcription allows the addition of reporter groups via iEDDA at internal sites of the target RNA [82].

3 Click Chemistry for Detection of Natural Oligonucleotides

The detection of specific, endogenous DNA and RNA sequences, especially in living cells, is challenging and can provide valuable information about their localization, expression levels and their lifetime. Being able to image specific DNA and RNA molecules in vivo in real-time could offer plenty of information on oligonucleotide synthesis, processing, transport and RNA degradation, with new insights for drug discovery and medical diagnostics. Various approaches aim to

label endogenous DNA and RNA with reporter groups such as fluorophores for detection. In this section, methods for labeling specific, unmodified DNA and RNA sequences in vitro (with potential for future in cell labeling) using click chemistry are briefly presented.

3.1 Site-Specific Labeling via DNA and RNA Recognizing Enzymes

DNA-specific enzymes, DNA methyltransferases (Mtases) can be employed to transfer activated groups sequence specifically to DNA from a cofactor, usually from modified *S*-adenosylmethionine (AdoMet) analogues. This approach has been used as a two-step labeling approach by first alkylating plasmid DNA via an alkyne derivatized AdoMet at the N^6-amino position of adenine and subsequent CuAAC reaction with an azide-modified fluorophore (Fig. 2) [83]. Three DNA methyltransferases belonging to the class of adenine-N^6 methyltransferases were identified from a screening of wild-type methyltransferases enzymes to successfully catalyze the alkyltransfer reaction: M.TaqI (target sequence 5′-TCGA-3′), M.XbaI (5′-TCTAGA-3′) and M.FokI (5′-GGATG-3′ and 5′-CATCC-3′) [83]. A major drawback of this approach is the fragmentation of the plasmid DNA during the CuAAC reaction, despite the presence of Cu^1 stabilizing ligands [83]. Copper-free click reactions such as SPAAC and iEDDA will certainly solve this issue.

A similar chemo-enzymatic approach has been used for fluorescent labeling of tRNA in vitro [84]. The tRNA-specific methyltransferase Trm1 was employed to transfer a pent-2-en-4-ynyl group from a modified AdoMet analogue to the N^2 position of guanosine 26 in $tRNA^{Phe}$ for subsequent CuAAC labeling [84].

Using the sequence-guided 2′-*O*-methyltransferase in box C/D ribonucleoprotein particles, sequence-specific RNA labeling via a two-step approach involving CuAAC click chemistry could also be achieved in vitro [85]. Notable for this approach is that the sequence-specificity is generated by the use of "guide RNAs," which allows labeling of various different RNA sequences by altering the guide sequence [85]. Despite the need for a complementary guide sequence to define the target RNA sequence, this method might in future show great potential for in cell labeling of RNA.

Rentmeister et al. developed a two-step approach to site-specifically label the 5′-cap of eukaryotic mRNAs via CuAAC [15] and SPAAC [14] click chemistry. For this, a variant of trimethylguanosine synthase 2 from *Giardia lamblia* is employed,

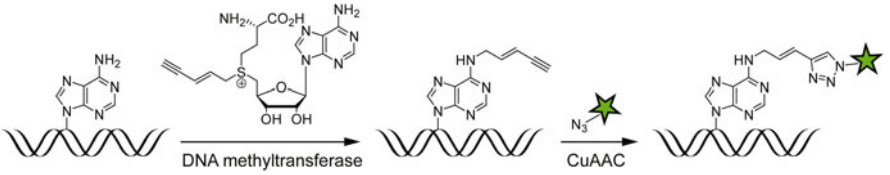

Fig. 2 Methyltransferase-directed N^6-adenine alkylation of DNA by 5′-[(*S*)-[(3*S*)-3-amino-3-carboxylpropyl](*E*)-pent-2-en-4-ynylsulfonio]-5′-deoxy-adenosine and subsequent CuAAC reaction with an azido-modified reporter group [83]

which recognizes the m^7G-cap structure of mRNA and transfers an alkyne group from a modified *S*-adenosylmethionine (AdoMet) analogue [15]. Further engineering of the enzyme allowed terminal azido-labeling of the 5′-cap for strain-promoted azide–alkyne cycloaddition (SPAAC) [14]. Recently, this method was extended to the transfer of a 4-vinylbenzyl group for inverse electron-demand Diels–Alder and photo-click reactions using a novel AdoMet-analogue [86]. The two-step labeling approach was demonstrated in eukaryotic cell lysate and in the future might be applicable for mRNA labeling in living cells, once complex issues, such as the synthesis of AdoMet analogues by cells and specificity of engineered enzymes for these analogues, have been solved.

3.2 Site-Specific RNA Labeling Using DNAzymes

Enzymatically catalyzed transfer of bioorthogonal groups to DNA and RNA is not solely limited to protein enzymes. Höbartner et al. [87] developed an in vitro approach for site-specific post-transcriptional RNA labeling using a deoxyribozyme (DNAzyme). Guanosine derivatives bearing bioorthogonal groups such as azide moieties were site-specifically transferred to the 2′-OH group of adenines in RNA oligonucleotides [88]. The DNAzyme recognizes the target nucleoside in the RNA substrate by hybridization. In the presence of Tb^{3+} as cofactor, a 2′,5′-phosphodiester bond is formed between the 2′-hydroxyl group of the adenosine in the target RNA and the α-phosphate of a 2′-azido–modified guanosine triphosphate derivative. Subsequent CuAAC click reaction allows the attachment of reporter groups such as fluorophores [88]. Long RNA oligonucleotides of up to 160 nucleotides are selectively labeled by this method [88]. This novel approach will certainly prove very useful for the selective labeling of long in vitro transcribed RNAs for biophysical studies.

4 Backbone Modifications and Click Nucleic Acid Ligation

Click chemistry has also been used to synthesize artificial DNA and RNA backbones via triazole linkages [89]. The first examples of an unnatural, biocompatible DNA backbone assembled by CuAAC click chemistry were demonstrated by Nakamura et al. [90] and Brown et al. [91]; the latter named this method “click nucleic acid ligation” [53, 89, 92]. PCR amplification of a DNA oligonucleotide linked via 3′-azido-dT and 5′-propargylamido-dT by CuAAC was demonstrated by Brown’s group in 2009; however, only the incorporation of a single thymidine residue was revealed by sequencing due to misreading of the triazole backbone by the DNA polymerase [91]. A more flexible second generation triazole linkage retaining the 3′-oxygen and more accurately mimicking the phosphate backbone could correctly be read by DNA polymerases. Both amplification in vitro by PCR as well as replication of a plasmid containing the triazole linkage in *E. coli* were demonstrated [93]. This approach was further extended to assemble long DNA oligonucleotides by multiple sequential ligation employing a masked azide approach and CuAAC and SPAAC reactions on solid phase [92]. The biocompatibility of the triazole linkage was further investigated in mammalian cells

using a click-linked gene encoding the fluorescent protein mCherry [94]. Error-free transcription of the click-linked gene was demonstrated [94].

For RNA, artificial self-cleaving hammerhead ribozymes with a triazole linkage at the active site are reported [26]. Interestingly, triazole backbone modifications in RNA by CuAAC and SPAAC chemical ligation of 3′-azide and 5′-cyclooctyne oligonucleotides can further be reverse transcribed into DNA while one nucleotide is omitted at the linkage [95].

In summary, click ligation of nucleic acids allows the assembly of long DNA or RNA strand with various reporter groups, which are, for example, incompatible with enzymatic ligation conditions or if an efficient enzymatic ligation is prevented by extensive secondary structures of the nucleic acid.

5 Unnatural Base Pairs for Enzymatic Site-Specific Labeling of Oligonucleotides Using Click Chemistry

Incorporation of site-specific anchor groups for the attachment of labels such as fluorogenic tags, spin labels or biotin is highly desirable for structural and functional studies on oligonucleotides, such as large, naturally occurring RNA molecules with usually complex foldings. These anchor groups need to be positioned at specific sites within the sequence, where they do not impede folding and function of the RNA molecule. However, this poses a problem for RNAs with sequences longer than 100 nucleotides, due to the limitations in solid phase RNA synthesis [3]. Complex ligation strategies are required to assembly RNA molecules with a length of 200–300 bases [96], a common size for various naturally occurring non-coding RNAs [97, 98], such as ribozymes or riboswitches. Ligation of highly structured RNAs often fails or results in low yields.

An enzymatic approach for site-specific incorporation of modified triphosphates overcomes this obstacle: Unnatural base pairs can be employed for the site-specific introduction of various functional groups into DNA and RNA via standard enzymatic reactions such as PCR and in vitro transcription. Several research groups have been concentrating on the development of a third, unnatural base pair over the last decade which can be PCR amplified and transcribed into RNA in vitro by standard polymerases with almost similar efficiency and fidelity compared to the natural base pairs [99–112].

A particular focus of interest lies on so-called hydrophobic base pairing systems (UBPs), which rely on shape complementary rather than hydrogen bonding interactions of the pairing bases, as first proposed by Kool et al. [113–115]. To date, different optimized hydrophobic base pairing systems exist, developed by the groups of Romesberg [107, 111, 112, 116–123] and Hirao [105, 106, 109, 110, 124–133]. Efficient PCR amplification of these base pairs was demonstrated; furthermore, in vitro transcription into RNA using the corresponding ribose building blocks has been reported [125, 127, 133–144]. Direct functionalization of these unnatural bases with biotin or fluorophores allows site-specific modification of PCR amplified DNA [143] and transcribed RNA oligonucleotides [136, 140, 145]. However, direct introduction of functional groups is limited by their size and shape and unnatural bases, bearing, for example, large fluorgenic tags are poorly incorporated by polymerases [143, 146].

Hirao et al. [136] demonstrated in 2005 that post-transcriptional labeling of a short 17mer RNA fragment containing an amino-modified unnatural 2-oxo-(1H)pyridine base is feasible. The unnatural base was site-specifically introduced via enzymatic in vitro transcription and subsequently reacted with the N-hydroxysuccinimidylester of 5-carboxyfluorescein or 5-carboxytetramethylrhodamine.

Analogously, the d5SICS–dNaM unnatural base pair developed by Romesberg et al. [107, 142] tolerates amino-modifications at both components of this unnatural base pair with sufficient efficiency in in vitro transcription reactions to allow post-synthetic RNA labeling using NHS-ester chemistry [111, 143]. As a first example of post-transcriptional functionalization of a long RNA, a site-specifically labeled 77 nucleotide amber suppressor tyrosyl tRNA from *Methanocaldococcus jannaschii* was synthesized via in vitro transcription and post-transcriptionally reacted with NHS-biotin [143].

5.1 Site-Specific DNA Functionalization with Alkyne Moieties

Functionalization of these unnatural nucleobases by 'clickable' moieties that are relatively small in size allows efficient incorporation during PCR and transcription, and subsequent attachment of various reporter groups. This versatile approach is particularly attractive for enzymatic synthesis of long DNA and RNA strands with site-specific modifications. Post-amplificational and transcriptional labeling via click reactions is more efficient, robust and particularly less pH-dependent compared to NHS-ester chemistry. Click functionalization of DNA strands containing unnatural base pairs was demonstrated by Romesberg et al. [120]. Amongst others, the d5SICS^{CO}–dNaM unnatural base pair (Fig. 3a) was employed to introduce an alkene moiety into PCR amplified DNA oligonucleotides at one and two specific positions. An azide-modified biotin tag as well as two molecules of the N-terminal Src homology 3 domain from the human CrkII adaptor protein (nSH3) were attached via Cu^{1}-mediated azide–alkyne click reaction after PCR amplification of the 133-nucleotide-long DNA fragment.

5.2 Site-Specific RNA Functionalization with Alkynes, Azides and Strained Alkenes

Site-specific RNA labeling using an alkyne-modified hydrophobic UBP termed Ds–Pa pair [125, 138, 140, 141] was first demonstrated by Hirao et al. [135]. The alkyne linker was attached to the Pa nucleobase for postsynthetic RNA labeling using copper-catalyzed azide–alkyne click chemistry (Fig. 3b, 1) [135], resulting in a versatile labeling method for large RNA molecules. Post-transcriptional attachment of large reporter groups such as fluorophores avoids reduced transcription efficiency and selectivity caused by these bulky functional groups [146]. By this method, a 75-nucleotide tRNA molecule was site-specifically alkyne-functionalized at position 33 and subsequently reacted with fluorescent azides or azide-modified biotin [135].

Introduction of strained unsaturated ring systems such as norbornene to an UBP allows bio-orthogonal inverse electron-demand Diels–Alder cycloadditions (iEDDA) [63, 66–69, 71–73, 75, 78, 80, 86, 147–150] on RNA with tetrazine

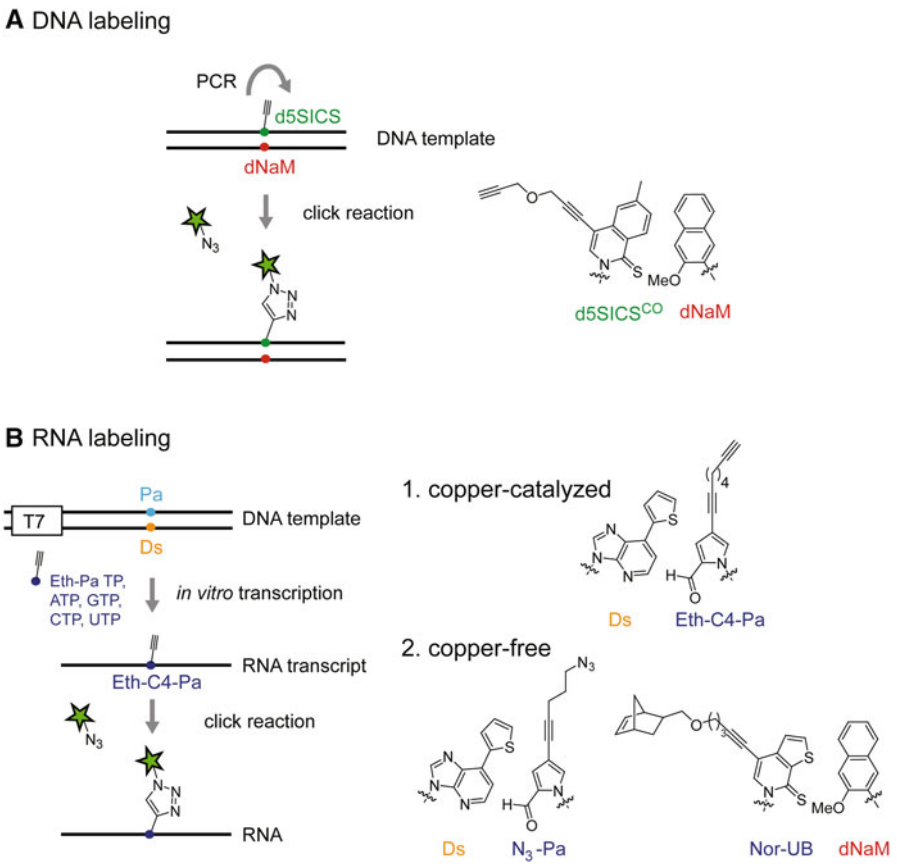

Fig. 3 Unnatural base pairs for enzymatic site-specific labeling of oligonucleotides using click chemistry. **a** DNA labeling using the unnatural d5SICS–dNaM base pair [120]. PCR amplification was carried out with an alkyne-modified d5SICS nucleoside that can be reacted with azide containing compounds. **b** RNA labeling via T7 in vitro transcription. The functionalized unnatural triphosphate is site-specifically incorporated into an RNA transcript in an in vitro transcription reaction and post-transcriptionally labeled via click chemistry. Triphosphates of the depicted unnatural bases for copper-catalyzed (Eth–C_4–Pa [135]) and copper-free click reactions (N_3–Pa [152] and Nor-UB [144]) have been developed

derivatives using non-denaturing conditions during the postsynthetic labeling step while maintaining fast reaction kinetics [66, 67, 80, 144, 150]. We modified one component of an unnatural base pair system developed by Romesberg et al. to introduce one or two norbornene moieties at predefined positions into RNA oligonucleotides in an in vitro transcription reaction using the RNA triphosphate Nor-UB and the unnatural base dNaM as template (Fig. 3b, 2). This allows functionalization of these RNA molecules directly after transcription with tetrazine derivatives containing for instance fluorophores or biotin. The native structure of the RNA will not be affected by the mild click reaction conditions, which can even be realized in a cellular context [66, 67, 80, 150]. This is of particular interest for labeling RNAs with

complex folding pathways, such as riboswitches, ribozymes or aptamers, which cannot be correctly refolded in vitro after a denaturing purification step [151].

Likewise, Hirao et al. applied copper free click chemistry for RNA functionalization via the 7-(2-thienyl)imidazo[4,5-b]pyridine (Ds) and pyrrole-2-carbaldehyde (Pa) unnatural base pair [152]. For this, an azide-modified Pa nucleobase (N_3–Pa) was incorporated as triphosphate in a T7 in vitro transcription reaction using Ds as corresponding nucleobase in the DNA template (Fig. 3b, 2). Post-transcriptional modification was achieved using fluorescent dibenzocyclooctyne (DIBO) derivatives in a strain-promoted azide–alkyne cycloaddition reaction. Transcription and efficient site-specific labeling of a 260mer RNA was demonstrated.

In summary, copper-catalyzed and copper-free click chemistry approaches combined with enzymatic site-specific introduction of reactive groups provide a powerful tool for labeling of long DNA and RNA molecules that cannot be synthesized via solid-phase synthesis. The concept of unnatural base pairs is no longer limited to in vitro applications. Romesberg et al. [112] demonstrated the faithful replication of a plasmid containing the d5SICS–dNAM base pair in *E. coli*. In principle, the site-specific functionalization of target RNA molecules expressed in a cell could be feasible in the future by using one of the described triphosphates. This would be an innovative and extremely useful tool for in vivo imaging of RNA, and could enable new insights into localization and transport of specific RNA molecules.

6 Metabolic Labeling of DNA and RNA

Copper-catalyzed and copper-free click chemistries have been used for metabolic labeling and fluorescent detection of DNA and RNA synthesis in vivo. Alkyne or alkene modifications on natural nucleosides are sufficiently small to allow efficient incorporation into DNA/RNA by cellular polymerases and circumvent drawbacks of immunohistochemical staining methods using 5-bromo-2′-deoxyuridine (Br-dU), such as the poor permeability of antibodies into various tissues.

A first example for metabolic labeling of DNA was the incorporation of 5-ethynyl-2′-deoxyuridine [12] (EdU, Fig. 4A) into cellular DNA and subsequent Cu^1-mediated azide–alkyne click reaction using fluorescent azides. [153] The labeling process of genomes of replicating cells is schematically described in Fig. 4B. Components for the EdU labeling strategy have been commercially available since 2008 to study cell proliferation and differentiation. In contrast to Br-dU labeling, no prior nucleic acid denaturation by acid, heat or DNase digestion to allow anti-Br-dU antibody binding is required for detection. The harsh conditions required for anti-Br-dU antibody detection can, for example, destroy tissue structures. The small sized fluorescent azides can, in contrast to antibodies, diffuse through tissues and react with alkyne moieties in the genome without DNA denaturation. For EdU labeling, cells are incubated with EdU followed by fixation and detergent permeabilization prior to the addition of the staining mixture containing the fluorescent azide $CuSO_4$ and sodium ascorbate [153]. The method has also been used for labeling in whole animals; for example, for studying cell proliferation in the nervous system of embryonic [154] and adult [155] mice.

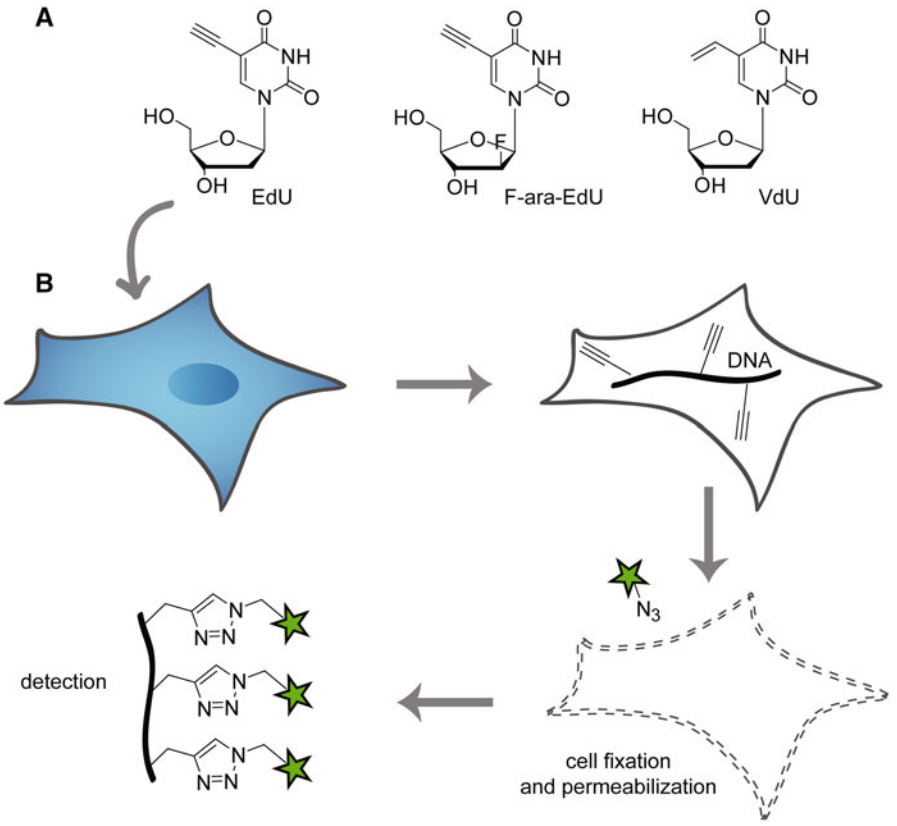

Fig. 4 Metabolic DNA labeling using alkyne- or alkene-modified nucleosides. *A* 5-Ethynyl-2′-deoxyuridine (EdU) [153], (2′*S*)-2′-deoxy-2′-fluoro-5-ethynyluridine (F-ara-EdU) [160] and 5-vinyl-2′-deoxyuridine (VdU) [167]. *B* Labeling strategy: cells are incubated with the alkyne-modified nucleoside EdU, which is incorporated into the cellular genome in proliferating cells. Subsequently, the cells are fixed, permeabilized and components for the Cu^{I}-mediated azide–alkyne click reaction with fluorescent azides are added. Detection can be carried out by flow cytometry or fluorescence microscopy

A similar strategy is applied for monitoring de novo RNA synthesis in cells and even in organs of whole animals using the corresponding ribose derivative, 5-ethynyl-uridine (EU) [156]. Significant labeling of cellular DNA is not observed, allowing RNA detection with high sensitivity using fluorescent azides.

A major drawback of metabolic labeling strategies using EdU is the cytotoxicity of EdU, which leads to DNA damage, cell cycle arrest and apoptosis [157–159]. Thus, the application of EdU in long-term labeling experiments is limited. Other alkyne-modified nucleosides have been developed and used in metabolic labeling experiments that exhibit lower cytotoxic effects. Interestingly, the D-arabinose configurated (2′*S*)-2′-deoxy-2′-fluoro-5-ethynyluridine (F-ara-EdU) (Fig. 4A) could be incoporated into cellular DNA with high efficiency, and possesses minimal impact on cellular proliferation [160]. Zebrafish embryogenesis was investigated over multiple days, demonstrating that successful long-term labeling is possible due

to the lowered cytotoxicity of F-ara-EdU [160]. Alkyne-modified cytidine (EdC [161, 162]), adenine (EdA [163]) and guanine (EdG [163]) derivatives with slightly reduced cytotoxicity compared to EdU have also been employed for metabolic DNA labeling in cells and zebrafish embryos and could be useful tools to study pyrimidine and purine nucleoside metabolism.

The ribose equivalent 5-ethynylcytidine (EC) proved to be a valuable tool for monitoring RNA synthesis in vitro and in vivo (mice), exhibiting similar sensitivity to EU and was faster metabolized than EU [8]. Interestingly, RNA labeling with the purine nucleoside 5-ethynyl-adenosine (EA) in contrast did result in unspecific labeling of both cytoplasm and nuclei, and is not suitable for the specific detection of RNA synthesis in cells [8].

EdC, F-ara-EdU and EdA, as well as the corresponding ribose nucleoside EU, have also been used for tracking viral DNA [164] and RNA [165] genomes, respectively, in host cells. The use of super-resolution microscopy techniques allowed detection at single viral genome sensitivity [164]. Pathogen specific DNA labeling of cells infected with Herpes Simplex Virus-1 (HSV-1) has recently been achieved with an alkyne-modified gemcitabine metabolite 2′-deoxy-2′,2′-difluoro-5-ethynyluridine [166]. This nucleoside derivative is metabolized by a viral low fidelity thymidine kinase resulting selective labeling of HSV-1 infected cells.

A disadvantage of all metabolic labeling strategies based on azide–alkyne click reactions is the required Cu(I) for catalysis, which degrades cellular DNA and RNA. In 2014, metabolic labeling of DNA could be achieved using the catalyst-free inverse electron demand Diels–Alder cycloaddition reaction between alkenes and tetrazines [167]. 5-Vinyl-2′-deoxyuridine (VdU, Fig. 4A) was metabolically incorporated into genomic DNA of HeLa cells and subsequently visualized with a fluorescent dipyridyl tetrazine derivative. Its genotoxicity is reduced compared to EdU [167]. Orthogonal labeling using VdU in combination with EdU/F-ara-dU and Br-dU, followed by staining with a tetrazine–fluorophore conjugate, a fluorescent azide and the Br-dU antibody is possible, allowing multi-color labeling to observe spatial and temporal distribution of genomic DNA during *S*-phase [167].

An azide-modified nucleoside has also been used for copper-free DNA detection with fluorescent cyclooctynes via strain-promoted azide–alkyne cycloaddition. 5-(Azidomethyl)-2′-deoxyuridine (AmdU) is, in contrast to various other azide containing nucleosides, stable in solution and metabolically stable and could be incorporated in cellular DNA [35]. Recently, this method has been extended for the detection of cellular RNA by 5-azidopropyl-modified UTP analogs [168].

Nucleosides modified with strained alkenes such as cyclopropenes, norbornenes or cyclooctenes have not been tested for metabolic labeling so far. These alkenes exhibit faster reaction rates with tetrazines than VdU; however, incorporation of the modified nucleosides into DNA by cellular enzymes might be limited due to the sterically demanding substituents. If successful, live-cell imaging of cellular DNA and RNA may be feasible in the future, using such nucleosides modified for fast iEDDA cycloaddition reactions with tetrazine derivatives. For this, tetrazine–fluorophore conjugates that exhibit significant turn-on fluorescence upon reaction with alkenes (usually with fluorophores emitting between 510 and 570 nm) have to be employed, to avoid strong background fluorescence.

7 Click-SELEX

In vitro selection (SELEX, Systematic Evolution of Ligands by Exponential Enrichment) has been employed for over a decade to evolve aptamers and artificial ribozymes with various catalytic functions [169, 170]. However, nucleic acids are, in contrast to proteins, limited to the four nucleobases, thus occupying a more narrow chemical space, which can limit successful SELEX experiments against particular targets. Modified nucleosides in nucleic acid libraries are useful tools in aptamer SELEX to expand the chemical space of DNA [171–174].

The introduction of functional groups on canonical nucleobases can be restrained, because these modified nucleobases need to be compatible with the enzymatic steps required in the SELEX process. A novel approach termed click-SELEX uses a modular strategy based on Cu^{1}-catalyzed azide–alkyne cycloaddition to generate modified nucleic acid libraries (Fig. 5) [175].

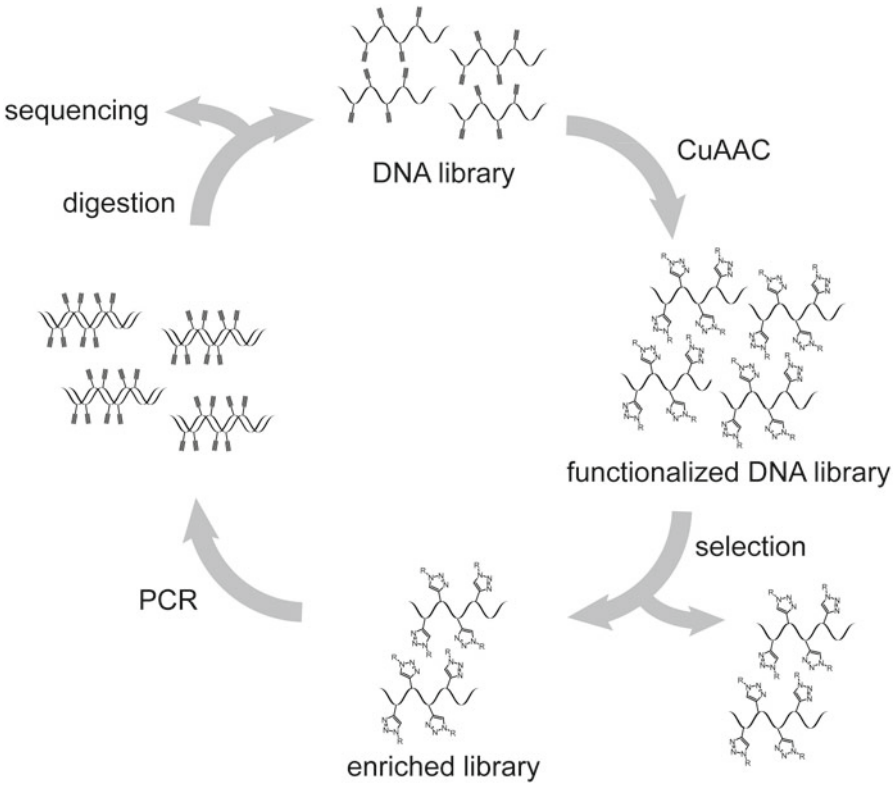

Fig. 5 Schematic representation of the click-SELEX process: An EdU containing DNA library is functionalized via CuAAC using an azide-modified molecule. The library is incubated with the target molecule, unbound sequences are removed and the enriched library is amplified by PCR using EdU triphosphate instead of the canonical thymidine to reintroduce the alkyne moiety. Single-stranded DNA is prepared by λ-exonuclease digestion. The modification can again be introduced by CuAAC for the next selection cycle. Adapted from [175]

Alkyne-modified libraries containing 5-ethynyl-2′-deoxyuridine [12] (EdU, Fig. 4A) instead of thymidine are prepared. Functionalization of the library is achieved by copper-catalyzed click reaction with azide containing compounds. As a proof of principle, 3-(2-azidoethyl)indole was used and successful selection was performed against cycle 3 GFP [175]. The amplification step after selection is carried out in the presence of EdU to restore an enriched alkyne-modified library for subsequent rounds of selection. At this point, correct amplification of the functionalized template strand by DNA polymerases is still a requirement for successful selection, and can restrict the size of the azide-modified compounds used.

In future, this might be achieved by the introduction of photocleavable linker molecules to remove large residues prior to PCR amplification [175]. Thus, various chemical moieties such as aromatic residues, amino acids or lipid modifications could be introduced in nucleic acid libraries by this method, allowing rapid access to modified libraries for aptamer selection and the selection of novel DNA catalysts.

8 Click Chemistry in Nanostructure Assembly

Click chemistry has proven to be a valuable tool to stabilize self-assembled nucleic acid nanostructures by covalent cross-links that are more stable to denaturing agents, or can, for example, be freeze-dried. Discussing various aspects of nanostructure stabilization will go beyond the scope of this chapter. Thus, only selected examples involving click chemistry for DNA nanostructure assembly and stabilization are presented.

Hexagonal DNA modules have been self-assembled and stabilized via CuAAC click chemistry by six simultaneous reactions, a method which is applicable for stabilization of various DNA nanostructures [176].

DNA nanopatterns on surfaces have been immobilized using click chemistry [177], and branched, Y-shaped DNA molecules can be prepared from tripropargylated oligonucleotides [178] by CuAAC click reactions, which are useful building blocks for higher DNA nanostructures. Moreover, SPAAC click chemistry in combination with orthogonal photochemical fixation has been used to synthesize oligomeric DNA scaffolds from cyclic DNA nanostructures which are stable towards denaturation and allow facile purification [179].

In a recent example, Cassinelli et al. [180] demonstrated that highly complex DNA-catenanes can be formed from 24 simultaneously interlocked DNA rings, resembling a chainmail architecture. Ring closure was achieved via CuAAC click chemistry [180]. Applications of this approach in nanobioelectronics, nanooptics or nanomedicine are imaginable.

9 Conclusion and Perspectives

In summary, click chemistry on nucleic acid has revolutionized the synthesis and assembly of chemically modified oligonucleotides for various biophysical and biochemical applications. In vitro preparation of labeled DNA and RNA via copper-catalyzed and copper-free click chemistry is well established to date.

Future applications might include progress in selection of functional nucleic acids (aptamers, ribozymes) with chemical entities not naturally present in DNA (or RNA), the preparation of even more complex nanomaterials built from nucleic acids, and certainly, progress in the development of novel approaches for site-specific labeling of nucleic acids in living cells or organisms to study the spatiotemporal distribution of specific DNA and RNA molecules.

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