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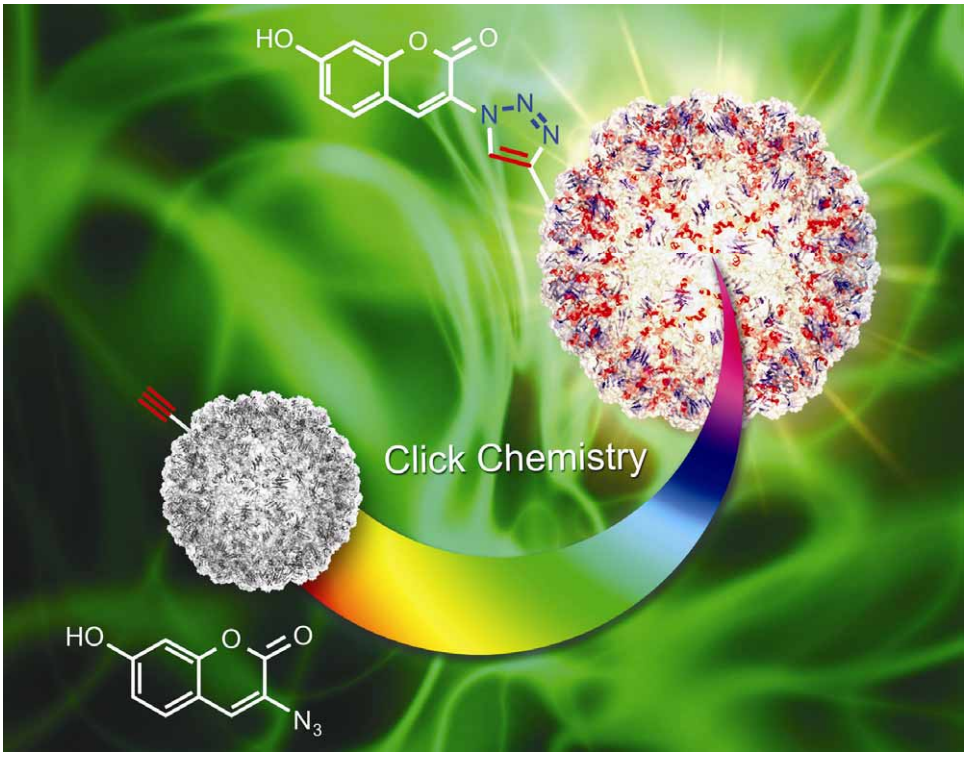

Click chemistry with DNA†

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The advent of click chemistry has led to an influx of new ideas in the nucleic acids field. The copper catalysed alkyne–azide cycloaddition (CuAAC) reaction is the method of choice for DNA click chemistry due to its remarkable efficiency. It has been used to label oligonucleotides with fluorescent dyes, sugars, peptides and other reporter groups, to cyclise DNA, to synthesise DNA catenanes, to join oligonucleotides to PNA, and to produce analogues of DNA with modified nucleobases and backbones. In this *critical review* we describe some of the pioneering work that has been carried out in this area (78 references).

Introduction

Click chemistry was developed to provide a simple method to join together organic molecules in high yields under mild conditions and in the presence of a diverse range of functional groups.[3] The best example of this new class of extremely efficient chemical reactions is the Cu^{I} catalysed [3+2] azide–alkyne cycloaddition (CuAAC) reaction.[1,2] There is a great deal of interest in developing new synthetic methods to construct chemically modified DNA oligonucleotides (ODNs) for biological and nanotechnological applications and it quickly became apparent to researchers in the nucleic acids field that the CuAAC reaction has great potential. The features of the click ligation reaction that are potentially useful in such applications are:

- Azides and alkynes can be attached to nucleic acids without greatly disturbing their biophysical properties.
- Azides and unactivated alkynes are almost entirely unreactive towards the functional groups normally encountered in nature; they react only with each other.
- The triazole unit is extremely stable, and is not toxic.

In this review we describe the use of click chemistry across the nucleic acids field, focusing on synthetic strategies and briefly describing some important practical applications. The basic CuAAC click reaction is shown in Fig. 1.

Oligonucleotide labelling with fluorophores and carbohydrates

A very early study on oligonucleotide labelling using 1,3-dipolar cycloaddition chemistry (Huisgen's AAC reaction[4–6]) involved the reaction of alkynyl 6-carboxyfluorescein (FAM) with azide-functionalised single-stranded (ss) ODNs to produce fluorescein-labelled ODNs in near quantitative yield.[7] The 5′-azido-oligonucleotides were made by labelling the equivalent 5′-aminohexyl oligonucleotides with succinimidyl 5-azidovalerate. The azide oligonucleotides were reacted with a 150-fold excess of alkynylamido-carboxyfluorescein in DMSO at 80 °C for 72 h. The long reaction time and high

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† Part of a themed issue reviewing the latest applications of click chemistry.

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Tom Brown

Tom Brown received his first degree and PhD at Bradford University after which he moved to Nottingham University, then to Oxford and Cambridge Universities to carry out post-doctoral research. He was then appointed to a lectureship at Edinburgh University where he was promoted to Reader then Professor of Nucleic Acids Chemistry. He moved to a Chair in Chemistry at Southampton University in 1995. His research interests centre on nucleic acids chemistry, structure, DNA sequence recognition and the application of oligonucleotide chemistry to biology and medicine. He is co-founder of three Biotech companies.

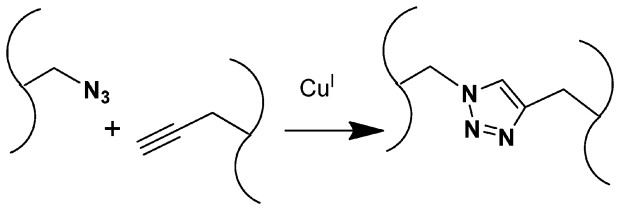


Fig. 1 The CuAAC reaction between an azide and a terminal alkyne to produce a 1,4-triazole.

temperature was necessary because the reaction was carried out in the absence of Cu^{I}. The fluorescein-labelled oligonucleotides were used as primers in DNA sequencing. Since then a more common approach to "click DNA labelling" has been to reverse the chemistry and conjugate alkyne-labelled DNA to azide-functionalized reporter groups. This is because alkynes are compatible with phosphoramidite oligonucleotide synthesis, whereas azides can potentially react with P^{III} (Staudinger reaction).[8] Carell *et al.* have used this principle to make seminal contributions in the field of DNA labelling, exploiting the remarkably efficient Cu^{I} catalysed [3 + 2] azide–alkyne cycloaddition (CuAAC) reaction.[1,2,9–11] This work is of great practical value because it can provide an efficient source of labelled DNA for use in biomedical applications, particularly in genetic analysis, genome screening and high-throughput DNA sequencing. Fluorescently tagged nucleotide building blocks are required for the synthesis of DNA probes which are used in many applications, for example fluorescent *in situ* hybridization (FISH). Until the advent of click chemistry such probes were usually made by reaction of amino-modified deoxynucleoside triphosphates with activated esters of fluorescent dyes, followed by enzymatic incorporation of the fluorescent triphosphates into DNA. However, the incorporation of bulky unnatural nucleotide triphosphates into DNA during PCR is a very inefficient process. Chemical synthesis of long strands of DNA containing such modifications is also fraught with problems caused by constraints on the length of DNA that can be produced by solid-phase synthesis, and by the instability of some fluorophores to the conditions of oligonucleotide deprotection. An alternative strategy is to incorporate simple sterically undemanding, potentially reactive chemical groups into DNA by enzymatic synthesis, and then to selectively react these with a variety of activated fluorescent labels. This requires a protocol in which suitably modified nucleoside triphosphate building blocks, or modified oligonucleotide primers are incorporated into DNA strands by PCR or other enzymatic methods. The ideal post-synthetic chemical functionalisation protocol must be highly efficient and specific, and should result in the quantitative incorporation of fluorophores into the DNA. The CuAAC reaction[1,12] fulfils these criteria. To establish this principle, Carell *et al.* prepared a series of alkyne-modified 16-mer ODNs of varying alkyne labelling density which contained the alkyne-modified deoxyuridines **1a** and **2a**. These were introduced *via* their phosphoramidites **1b** and **2b** during solid-phase oligonucleotide synthesis.[10] The efficiency of the click labelling reaction on these oligonucleotides was investigated using various azide-labelled reporter groups (Fig. 2).

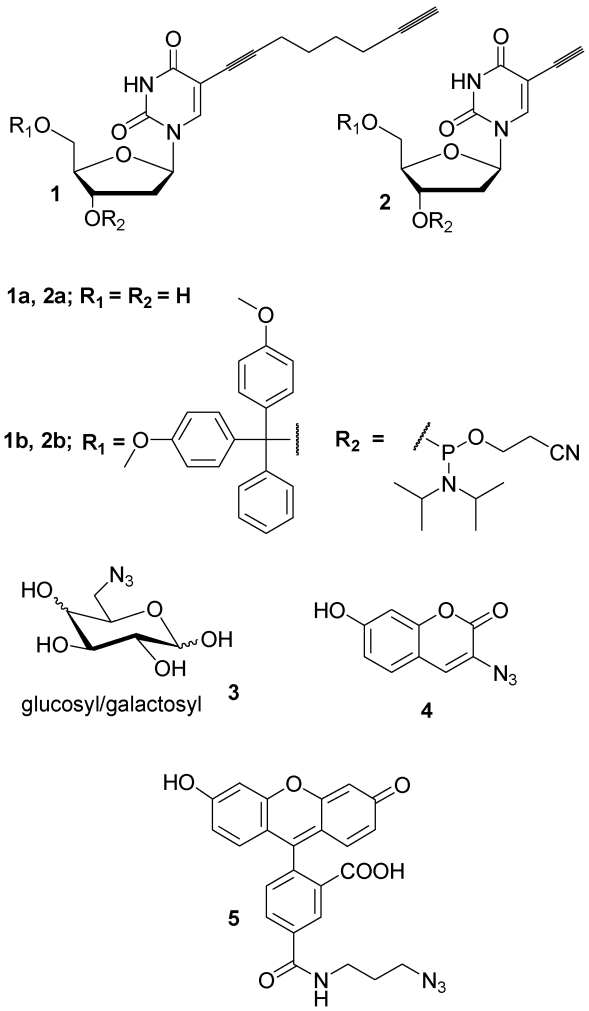


Fig. 2 Alkyne precursors, monomers and azide labels used in click oligonucleotide labelling by Carell *et al.*[10]

In nucleoside analogue **1a** the alkyne function is separated from the uracil base by a hexynyl spacer to circumvent potential steric problems with the high-density labelling of DNA which might be expected with ethynyl dU **2a** in which the acetylene is attached directly to the heterocyclic nucleobase. Up to six additions of alkyne deoxyuridines **1a** and **2a**, either contiguous or separated by other normal nucleotides, were inserted into the oligonucleotides. Three representative small molecule azide labels were chosen (**3–5**), one is a sugar with a masked aldehyde function suitable for use in silver staining, the second is a masked fluorophore (a coumarin) which becomes highly fluorescent upon triazole formation, and the third is a derivative of fluorescein (FAM), the most common fluorophore used in biological studies. Using the Cu^{I}-stabilizing ligand [tris(benzyltriazolylmethyl)-amine],[13] full conversion of the oligonucleotides containing a single alkyne into the respective triazole products was observed with all three azides. The choice of alkyne is important to achieve an efficient click reaction, and in the case of multiply-labelled oligonucleotides, octadiynyl dU gave quantitative labelling whereas ethynyl dU gave partial labelling, presumably due to steric hindrance. With the above labelling

methodology established, its power was further demonstrated by incorporating PCR primers containing two alkyne labels into a range of PCR products which were then efficiently labelled with fluorescein azide **5**. In general, the use of a Cu^{I}-stabilizing ligand in the CuAAC reaction for DNA labelling is recommended, because it increases reaction rate,[13] and protects the DNA from degradation. In the absence of a suitable Cu^{I}-binding ligand the half life of DNA in the CuAAC reaction is just a few minutes.[14]

Adding labels to PCR products *via* the oligonucleotide primers has limitations, as the labels can only be added in the primer regions. This is of limited use if long, densely-labelled PCR products are required. An alternative strategy is to incorporate the alkyne or azide functions into DNA by enzymatic methods *via* deoxynucleoside triphosphates. This has the distinct advantage of allowing very large numbers of labels to be distributed throughout the DNA strands during PCR. To achieve this aim, a combination of "click chemistry" and PCR has been used to introduce alkyne-modified deoxynucleoside triphosphates into DNA.[15] A series of PCR products of different lengths were prepared using a variety of alkyne- and azide-containing pyrimidine deoxynucleotide triphosphates (dNTPs) **6–10** (Fig. 3). Starting with short PCR products (300 bases), it was found that 5-octadiynyl dUTP **6** and 5-octadiynyl dCTP **9** were incorporated efficiently provided that the "type B" thermostable polymerases *Pyrococcus woesei* (Pwo), *Thermococcus litoralis* (Deep Vent *exo*-), or *Thermococcus kodakaraensis* (KOD XL) were used. Interestingly, the "A family" polymerase *Thermus aquaticus* (Taq) was found to be unsuccessful in this application. The inefficient incorporation of triphosphates **8** and **10** with any polymerase shows that a small modification of the structure of the dNTP can lead to a catastrophic failure. In the case of triphosphate **10** there are clear precedents; dNTPs with a single bond joining a side-chain to the 5-position of the pyrimidine base are known to be poorly accepted by polymerases.[16,17] Unfortunately, the analogue of **10** with an internal triple bond at the 5-position cannot be used, as it undergoes an intramolecular reaction with its own azide.

After completion of this preliminary work, PCR amplification of a longer DNA fragment with 900 base pairs was carried out. This longer PCR product comprised 551 exchangeable thymidines and 352 exchangeable cytidines in the duplex. Careful balancing of the PCR additives and cycling temperatures was necessary for efficient amplification, including elongation of the PCR cycle time, and addition of 4% dimethyl sulfoxide (DMSO) to decrease the annealing temperature of all DNA hybrids formed during the PCR. Only KOD XL polymerase was able to generate full-length amplicons with octadiynyl dU triphosphate **6**, and this was less well incorporated than the corresponding dC analogue **9**. The incorporation of modified triphosphate **9** into DNA strands 2000 base pairs (bp) in length, which comprise 887 cytidines, was also investigated. Careful optimization of the PCR protocol was required to reduce the unspecific binding of the primer, probably caused by the well-known increase in T_m of duplexes containing 5-alkyne modified nucleosides.[18,19] As above, DMSO was added and the denaturing temperature was set to 99 °C. The correct PCR product was only formed in a small range

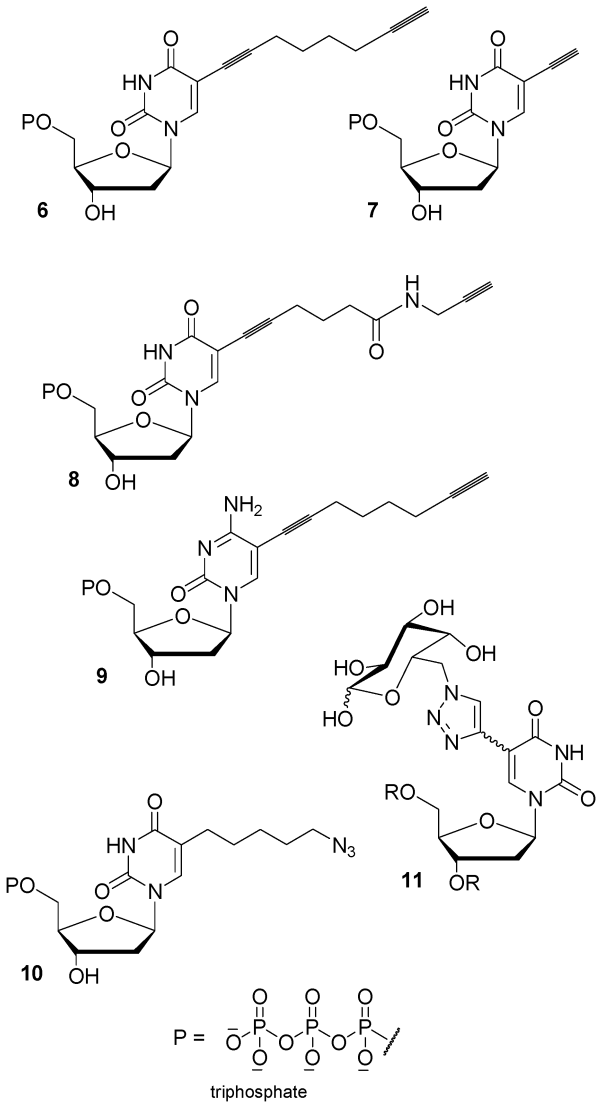


Fig. 3 Nucleoside triphosphates, azide labels and triazole products used for PCR-based click DNA labelling.

of annealing temperatures around 59 °C, as at higher temperatures shorter amplicons were synthesised. The final optimized protocol allowed the creation of a 2000-base-pair PCR product with 887 alkynes attached to the modified deoxycytidines. The alkyne-labelled PCR products were reacted with sugar azide **3** to produce sugar-triazole labelled amplicons containing nucleoside **11**. A remarkably high ($\geq$ 95%) clicking efficiency was achieved, testament to the high reactivity and selectivity of the CuAAC reaction, even on such large biomolecules. The octadiyne-modified dC triphosphate **9** gave particularly good results, no alkyne starting nucleoside being detected after the click reaction. This study also revealed that consecutive repeats of alkyne deoxynucleotides are incorporated inefficiently into PCR products. As in previous studies, a Cu^{I}-binding ligand was used throughout this work, and under these conditions the long DNA templates and PCR products were stable. Recently similar work has been carried out on alkyne-labelled purine deoxynucleotide triphosphates.[20]

The efficiency of the click reaction on PCR products containing the shortest alkynyl nucleoside studied (ethynyl dU) was investigated in detail.[11] The decreased click reaction yield for DNA modified with ethynyl dUTP **7** relative to its long-chain octadiynyl analogue **6** could be caused by steric shielding of the alkyne group due to the proximity of the nucleobase. In order to evaluate this possible steric effect, the authors set out to compare the click reaction efficiency of double-stranded (dsDNA) with single-stranded (ssDNA) using a 300-mer DNA construct.[11] To this end, they prepared ssDNA labelled with ethynyl dUTP **7** by an enzymatic approach. First, dsDNA was produced by a PCR reaction in which one of the two primers was phosphorylated at the 5′-position. The resulting DNA duplex was treated with the enzyme lambda exonuclease, which digests only the phosphorylated strand, leaving the complementary strand intact. On average every fourth nucleobase of the sequence was alkyne-modified and constituted a potential site for the click reaction. If steric factors were to be neglected, one would expect a comparable click reaction yield for all three substrates labelled with ethynyl dU (*i.e.* each individual single strand and the corresponding duplex). In order to examine the possible differences in click reaction yield between single- and double-stranded DNA, these substrates were subjected to a click reaction with sugar azide **3**, then enzymatically digested to the free nucleosides which were analyzed by HPLC-MS/MS. This study showed that the single-stranded DNA reacted in significantly higher yield (92%) than double-stranded DNA (78%). This difference was attributed to steric hindrance in the major groove of the duplex at the site of the alkyne moiety. An increase in the catalyst concentration had only a small effect on the efficiency of the reaction, but increasing reaction time or azide concentration had a more beneficial effect. The authors commented that in the case of difficult click labelling reactions on DNA, increasing the azide concentration should be the first step of an optimization process.

Important work on oligonucleotide labelling has also been carried out by the Seela group. ODNs containing alkyne-labelled 7-deazapurines **12** and **13** and pyrimidines **14** and **1a** have been studied (Fig. 4).[21] The required phosphoramidites were synthesised and then used in solid-phase oligonucleotide synthesis. The octa-1,7-diynyl nucleoside precursors were obtained from their corresponding iodo-derivatives using the palladium-assisted Sonogashira cross-coupling reaction. Melting experiments indicated that in all cases the octa-1,7-diynyl nucleosides have a positive influence on DNA duplex stability, more so than oligonucleotides functionalised with single alkynes.[22,23] Oligonucleotides containing the octadiynyl derivative of dU were also used in CuAAC reactions on solid-phase and in solution to link the DNA strands to 3′-azido-thymidine (AZT) and to an aromatic azide-compound.[22] In a related study, 7-alkynyl-7-deaza-2′-deoxyinosine was synthesised and used as a universal nucleoside.[24] The hypoxanthine base of deoxyinosine, which lacks the 2-amino-group of guanine, can form stable base pairs with guanine, thymidine and adenine, and to a lesser extent with cytosine.[25] As such it has been used as a "universal base" in degenerate hybridization probes and primers. Ideally, a universal DNA base should be able to pair efficiently to all four Watson–Crick bases, and the more neutral its base pairing properties the better. A pyrrolo dC analogue of deoxycytidine with a terminal alkyne was also prepared and incorporated into oligonucleotides.[26] The terminal alkyne residues of oligonucleotides bearing the above alkyne and octadiynyl groups[27] were selectively conjugated by the CuAAC reaction to the non-fluorescent 3-azido-7-hydroxycoumarin **4** to give strongly fluorescent 1,2,3-triazole conjugates (Scheme 1). The fluorescence properties of oligonucleotides with these covalently linked coumarin-nucleobases displayed the expected pH-dependence of fluorescence intensity.[27] The generation of fluorescence by the "click" reaction can be used for the visualization of DNA in free solution or embedded in DNA–protein complexes, and can also be used for the labelling and visualization of biomolecules *in vivo*.

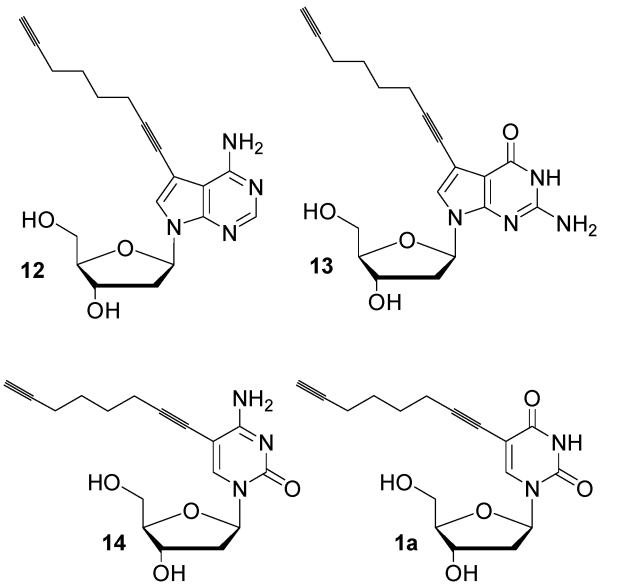
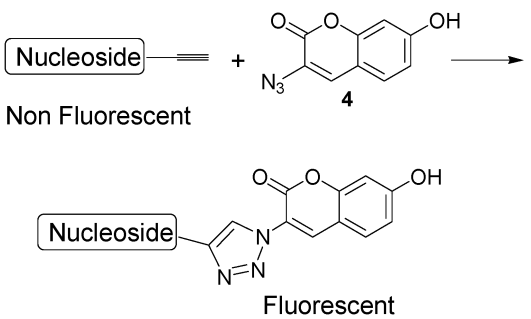
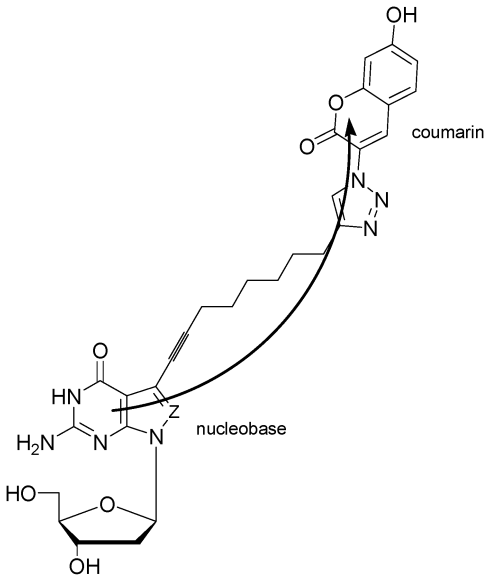


Fig. 4 Alkynyl dA, dG, dC and dU nucleosides used by Seela *et al.* in oligonucleotide labelling.

Oligonucleotides containing 7-(octa-1,7-diynyl) derivatives of 7-deaza-2′-deoxyguanosine were also conjugated to 3-azido-7-hydroxycoumarin by the click reaction (Fig. 5).[28] Pyrazolo[3,4-*d*]pyrimidine nucleoside derivative **15** shows a much higher fluorescence intensity than that of the corresponding pyrrolo[2,3-*d*]pyrimidine derivative **16**. The quenching in the dye conjugate **16** was found to be stronger with monomeric nucleoside conjugates than in single-stranded or duplex DNA. Nucleobase–dye contacts are suggested to be responsible for the

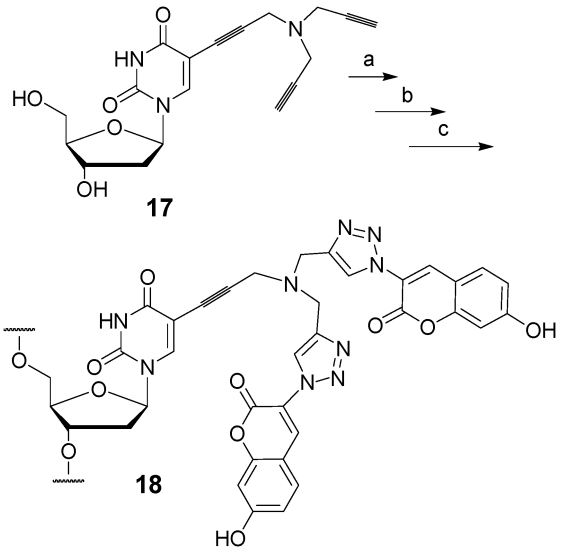


Scheme 1 Click chemistry to generate fluorescent nucleoside analogues from non-fluorescent precursors.



15 Z = N, no electron transfer
16 Z = C, electron transfer

Fig. 5 Electron transfer studies with pyrrolo/pyrazolopyrimidines and attached triazole coumarin.



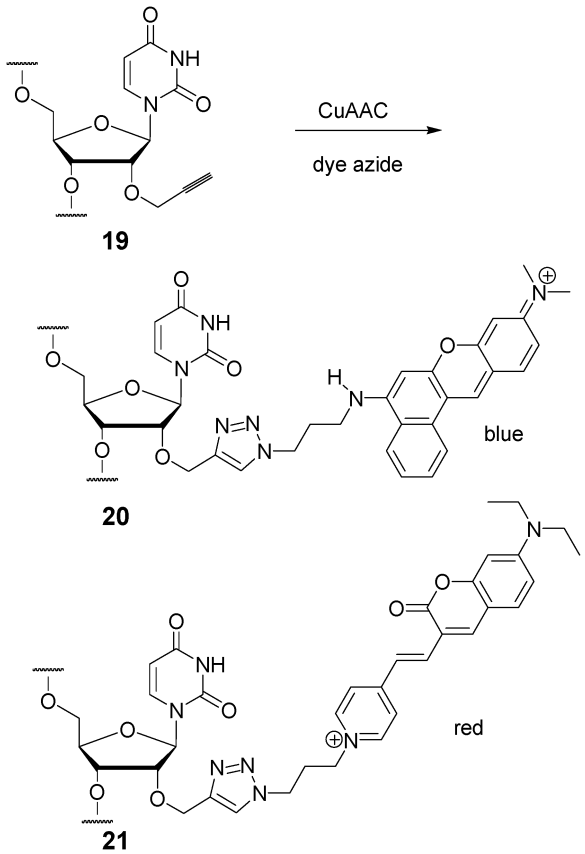
Scheme 2 (a). Convert to phosphoramidite monomer. (b). Incorporate into oligonucleotide. (c). Label with 3-azido-7-hydroxycoumarin by CuAAC reaction.

quenching (Fig. 5). These interactions are more favourable in the monomeric state than in the DNA chain when the nucleobase is part of the stack and is therefore held away from the coumarin. The side chains with the bulky dye conjugates are well accommodated in DNA duplexes thereby forming hybrids which are slightly more stable than canonical DNA.

The Seela group has also used click chemistry to introduce two fluorescent labels into a single thymidine unit.[29] Tripropargylamine-dU **17** was synthesised by Sonogashira chemistry, converted to a phosphoramidite monomer, incorporated into an oligonucleotide and clicked to non-fluorescent 3-azido-7-hydroxycoumarin by the CuAAC reaction to give **18** (Scheme 2). The resultant bis-coumarin labelled oligonucleotide was less fluorescent than oligonucleotides labelled with a single coumarin triazolide. This is not surprising, as self-quenching between the fluorophores would be expected to occur. The tripropargyl dU was found to slightly stabilize DNA duplexes, rather like the well known stabilisation provided by a propynyl substituent.[19,30]

As discussed earlier, some fluorophores are labile in basic conditions, making them unsuitable for incorporation into oligonucleotides during solid-phase synthesis. Deprotection of the ODN with ammonia or reagents such as methylamine or potassium carbonate would destroy these fluorophores. A better synthetic strategy is to introduce sensitive fluorophores post-synthetically, *e.g.* by reaction of an alkyne-labelled ODN with an azide derivative of the fluorescent dye. This can also be done by reaction of amino-labelled ODNs with active esters of fluorescent dyes in aqueous buffer, but this amide bond forming process is less efficient than the CuAAC reaction, and active esters are readily hydrolysed in aqueous media. In contrast, alkynes and azides are very stable functional groups except when reacting with each other. A suitable position for label attachment to DNA is the 2-position of the ribose sugar as in compound **19** (Scheme 3), as substitution here does not affect duplex stability greatly. Wagenknecht *et al.* have used this strategy[31] to incorporate alkyne functions into oligonucleotides and label them with dye azides to give oligonucleotides **20** (blue) and **21** (red).

A versatile approach based on microwave assisted click chemistry and H-phosphonate oligonucleotide synthesis has been developed for the multiple labelling of oligonucleotides with sugars.[32] First, three *para*-disubstituted cyclohexyl linkers were added by means of an H-phosphonate monoester derivative to a solid supported dodecathymidine oligonucleotide (T_{12}) which had been prepared by solid-phase phosphoramidite chemistry (Scheme 4). The H-phosphonate diester linkages were then oxidized in the presence of propargylamine which resulted in the incorporation of the propargyl group by formation of a phosphoramidate linkage. Three repeats of galactosyl azide **22** were then conjugated to the solid-supported tri-alkyne-modified T_{12} in a CuAAC reaction which was assisted by the use of microwave irradiation. In the cycloaddition reaction 3.3 molar equivalents of azide per alkyne residue were used in the presence of $CuSO_4$–sodium ascorbate in water–methanol to yield the solid-supported protected trigalactosyl oligonucleotide product. Subsequent treatment of the carbohydrate-labelled oligonucleotide with aqueous ammonia afforded the fully deprotected trigalactosylated T_{12} oligonucleotide **23**. The same group has also described the synthesis of propargylated pentaerythrityl phosphodiester oligomers (PePOs) using a bis-propargylated pentaerythritol-based phosphoramidite. An azido fucose derivative was reacted under "click" chemistry conditions activated by microwaves to construct a series of glycosylated PePOs bearing up to ten L-fucose residues. Binding to a fucose-specific bacterial lectin was determined by an enzyme-linked lectin amplification competition assay. The IC_{50} values were 10–20 times better than for monovalent L-fucose and suggest a "macromolecular" rather than a "cluster" effect.[33]

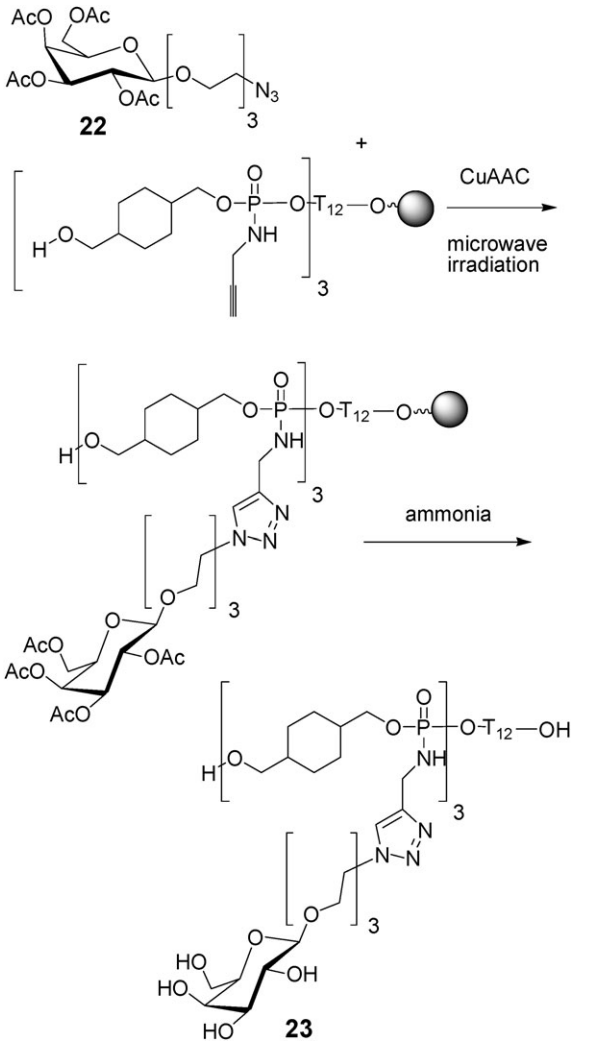


Scheme 3 Dye labelling at the 2-position of ribose in ODNs using the CuAAC reaction.

Glyco-oligonucleotide conjugates, each exhibiting two mannose and two galactose residues, were efficiently synthesised by two successive microwave-assisted 1,3-dipolar cycloadditions.[34] Two phosphoramidite derivatives were used: one bearing a bromoalkyl group as a precursor to azide functionalisation, and another bearing a propargyl group. After a first cycloaddition with a mannosyl-azide derivative, the bromine atoms were substituted with NaN_3 and a second click reaction was performed with a 1′-*O*-propargylgalactose, affording the heteroglyco-oligonucleotide conjugate.

Oligonucleotide immobilization

A procedure has been described to immobilize oligonucleotides on glass substrates in well-defined micropatterns by microcontact printing with a negatively charged, oxidized PDMS [poly(dimethylsiloxane)] stamp and positively charged dendrimers (polypropyleneimine tetrahexacontaamine dendrimers) which possess a large number of amino groups at the periphery.[35] The oligonucleotides were efficiently immobilized by "click" chemistry which was induced by microcontact printing (Fig. 6). The oligonucleotide immobilization is an irreversible one-step reaction. Alkyne-modified oligonucleotides were applied onto an azide-terminated glass slide under the confinement of the dendrimer-modified stamp without Cu^I catalysis. Oligonucleotides with the alkyne-modification



Scheme 4 Labelling oligonucleotides with carbohydrates *via* click chemistry.

at the 5′ terminus hybridized selectively with full-length complementary targets whereas strands with more than one acetylene linker did not hybridize with complementary strands due to steric hindrance at the surface. In this study the internal alkyne ODNs were made by solid-phase incorporation of octadiynyl dU phosphoramidite, and the 5′-alkyne was added using a phosphoramidite monomer based on octadiyne linked to a propanediol moiety.

A fluorogenic azidocoumarin click reaction has been used to verify the immobilisation of PNA and to construct PNA microarrays which were used for diagnosis of mutations in the breast cancer susceptibility gene BRCA 1.[36] Click chemistry has also been used to link DNA to hard (glass, silicon) and soft (polymeric) substrates. The approach is based on an alkyne end-functional diblock copolymer that self-assembles as a monolayer on the surface, directing the alkyne functions to the surface. Azide-functionalised DNA was then linked to the alkynes using a click reaction.[37] The azide-labelled ODNs used in this study were prepared by reacting 5′-aminohexyl ODNs with 6-azidohexanoic acid succinimidyl ester.

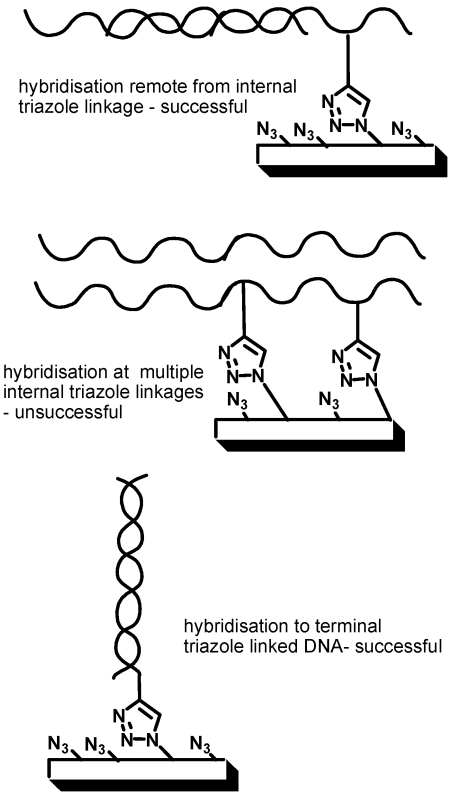


Fig. 6 Hybridisation efficiency studies on surface-bound DNA strands.

Oligonucleotide metallization

DNA is an excellent material for the preparation of nano- and microscale assemblies which have potential uses as nanoelectronic devices. For such applications DNA metallization procedures have been developed with the aim of increasing the conductivity of DNA nanostructures, thereby enabling them to be used as molecular wires. The metallization process, which involves the chemical reduction of DNA-complexed metal salts (Ag, Pd, Pt and Cu), results in uniform non-sequence-dependent metallized DNA architectures. However, for the construction of DNA-based electronic devices, a more selective protocol that allows sequence-selective metallization of DNA is required. Such a method has recently been developed, and involved the deposition of Ag^0 around aldehyde-modified DNA.[9] The method requires incorporation of acetylene-containing nucleotide triphosphates using DNA polymerases followed by a click reaction that can be performed directly on a polyacrylamide gel (Fig. 7). Using this method, Ag^0 deposition is confined only to the modified DNA. The ability to insert the acetylene labels enzymatically facilitates the synthesis of long DNA wires and offers the possibility to exploit molecular biology tools to construct conductive DNA nanodevices.

The same group has also used click chemistry to produce bimetallic Au/Ag nanowires,[38] and chain-like assembles of gold nanoparticles by growth on artificial DNA templates.[39] An ultrasensitive method has also been developed[40] for the detection of DNA and RNA in the diagnosis of genetically related diseases such as cancer. Future advancements in personalized medicine are critically dependent on the development of such

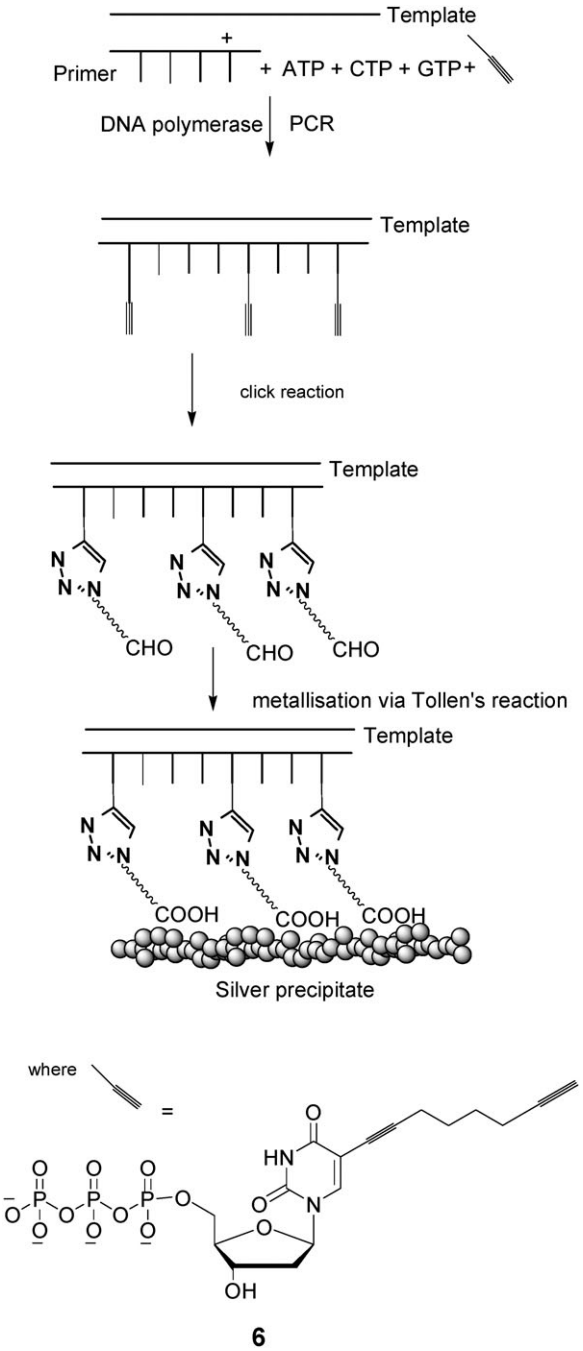


Fig. 7 Directed DNA metallization *via* click chemistry to create nano- and microscale assemblies.

reliable ultrasensitive DNA detection methods and the "DNA photography" method reported in this paper provides a potential solution to this problem. It is a simple and efficient method for ultrasensitive DNA detection in the femto- to attomole (10^{-18} mol) range based on an amplification process involving black and white photography.

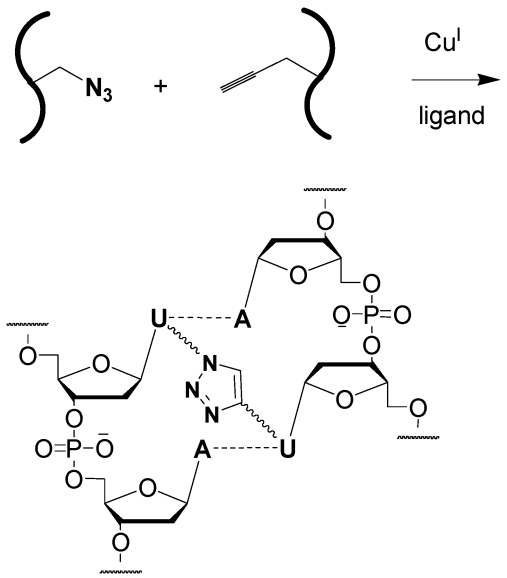
Oligonucleotide cross-linking

Click chemistry has been used to covalently cross-link complementary DNA strands across the major groove between

uracil bases to form very stable duplexes (Scheme 5).[41] For this purpose a single deoxyuridine (dU) nucleoside modified with a terminal alkyne was incorporated into a 14-mer DNA strand during solid-phase synthesis using either 5-ethynyl or 5-(octa-1,7-diynyl)- 2′-deoxyuridine phosphoramidite building blocks **1b** or **2b** (Fig. 8). Ethynyl dU[42] has the closest possible attachment of the alkyne to the nucleobase, and octadiynyl dU[10] has a flexible linker between the base and the terminal alkyne.

An azide-modified dU was incorporated in the sequence of the complementary strand. The synthesis of these azide oligonucleotides was achieved by incorporation of protected amino-modified nucleoside phosphoramidites **26c** and **27c** during solid-phase synthesis followed by post-synthetic derivatisation of the amines in aqueous buffer with the NHS-ester of 4-azidobutyric acid **24** or 6-azidohexanoic acid **25**. This provided DNA strands containing four different azides with varying linker lengths (10, 12, 15 and 17 atoms). The click reaction with ethynyl dU and azide with a 15 atom spacer was not efficient, probably due to steric hindrance at the alkyne. Reaction with octadiynyl dU and the same azide oligonucleotide was essentially quantitative, as in this case the steric environment was suitable. A similar observation on the reactivity of alkynyl deoxyuridines was made by Carell *et al.* when using the CuAAC reaction for post-synthetic oligonucleotide labelling.[10]

In the most favourable cases the templated CuAAC reaction between complementary DNA strands was extremely fast and was complete within 5 min. This demonstrates the remarkable speed of the CuAAC reaction when the two reactants are held in close proximity. The CD spectra of the double stranded products confirmed the presence of helically stacked nucleotides consistent with B-DNA, indicating that the presence of the triazole linker in the major groove of the cross-linked duplexes has no significant effect on DNA conformation. The thermodynamic stability of the cross-linked duplexes was measured by UV and fluorescence melting to reveal the effects of the various triazole cross-links. The melting temperatures of all cross-linked duplexes were much higher than the non-cross-linked controls. This is because their inter-strand interaction is intramolecular and close contact between complementary DNA strands favours base pair formation relative to hydrogen bonding between the bases and the surrounding water molecules. The magnitude of the increase in stability was in some cases more than 30 °C.



Scheme 5 Inter-strand duplex cross-linking between azide and alkyne-modified uracil bases in complementary oligonucleotides.

A combination of photochemical cross-linking and the CuAAC reaction has been used to develop a new method of genetic analysis (Fig. 9). The nucleoside phosphoramidite monomer of 5-ethynylvinyl-2′-deoxyuridine (^{EV}U) **28** was incorporated into an ODN[43,44] and the Huisgen cycloaddition between the ODN-^{EV}U and four commercially available aromatic organic azides was carried out to give triazoles **29a–d**. The resulting triazole ring-containing ODNs were subjected to photoligation with oligonucleotides with 3′-cytosine bases by irradiation at 366 nm in the presence of a template ODN to hold the two participating ODNs in close proximity. HPLC analysis indicated clean and efficient formation of ligated ODN. The photoligation rates with ODN-^{BTV}U were more rapid than those with the control non-triazole ODN-^{CV}U derived from **30**, indicating that the triazole rings, which have electron-donating characters, accelerate the photoligation reaction. The authors reported that ligation rates with ODN-^{PTV}U were more rapid than those with ODN-^{BTV}U because the phenyl system is more highly conjugated than the benzyl system. Moreover, the photoligation rates with ODN-^{MPTV}U and ODN-^{NTV}U, which have more strongly electron-donating substituents, were even more rapid. The time needed for 50% photoligation with ODN-^{NTV}U was one-fourth of the time with ODN-^{CV}U, and photoligation on a timescale of seconds was possible with ^{NTV}U. Similar photoligations were successful on systems in which a thymine base was cross-linked to the vinyltriazolyl dU-derivatised ODNs.

As a demonstration of a practical application of the above methodology, a model single nucleotide polymorphism (SNP) analysis was carried out. The authors synthesised a probe ODN containing a naphthyltriazole moiety and biotin on deoxyuridine ^{BNTV}U **31**. A DNA glass chip was constructed by attaching amino-labelled ODNs onto an aldehyde-modified glass surface. The chip was spotted with a target ODN which was irradiated and cross-linked to the probe, using a complementary ODN to template the reaction. A streptavidin–Cy3 conjugate was then added to the surface to bind to the biotinylated cross-linked ODN. Strong fluorescence was obtained from the photoligated product in the completely complementary case, showing a measured rate 10^3 times higher than with a single mismatch in the target sequence. Mutation detection by traditional methods shows only hybridization specificity, so the loss of DNA duplexes of matched sequences, particularly during the necessary washing steps, together with incomplete washing out of the mismatched sequences, decreases selectivity. In contrast, the fluorescence image generated after the photochemical ligation method showed no loss of the biotin-tagged probe, due to the covalent bonding between the capture and probe strands. Moreover, mismatched duplexes that could

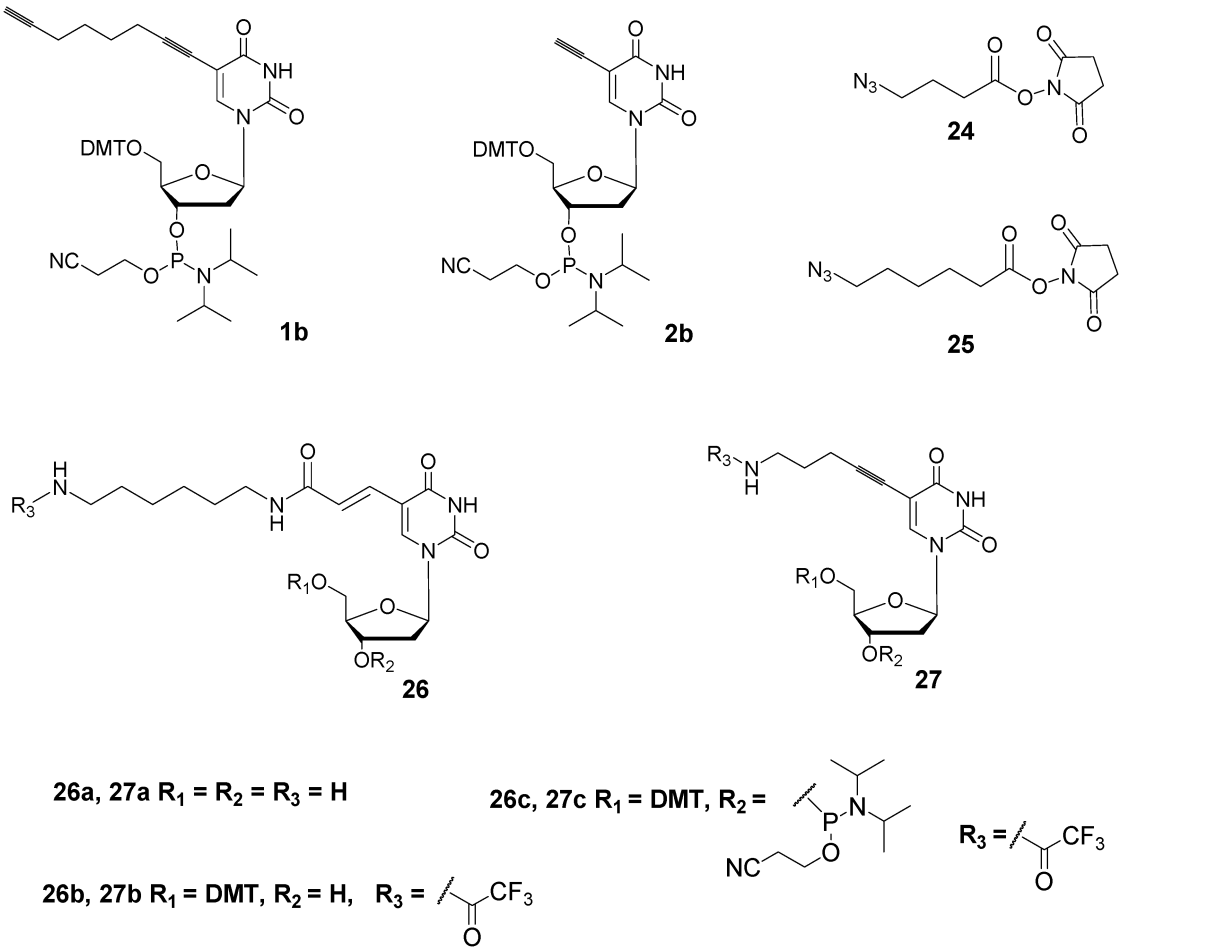


Fig. 8 Alkyne, amine and azide reagents, nucleosides and phosphoramidite monomers used in DNA double strand cross-linking by click chemistry.

potentially give rise to an unwanted background fluorescence image were eliminated completely by the high temperatures employed in the wash steps prior to photoligation.

As illustrated above, the attachment of labels onto DNA is of utmost importance in biomedical applications and is valuable in the construction of DNA-based functional nanomaterials. The CuAAC reaction allows the virtually unlimited functionalisation of both small synthetic oligonucleotides and large gene fragments with unprecedented efficiency. This emerging technology has recently been reviewed.[45]

Artificial DNA: oligonucleotide base and backbone analogues

A number of DNA analogues have been synthesised using click chemistry, in which the nucleic acid structure has been modified to incorporate triazole units. In one study, oligonucleotides **32b** containing a single addition of 1-ethynyl-2-deoxy-*b*-D-ribofuranose **32a** were reacted with a series of azides using the CuAAC reaction to produce ODNs containing artificial triazole nucleobases (Scheme 6).[46] In the best cases the click reactions were essentially quantitative. None of the artificial nucleobases produced stable DNA duplexes with complementary ODNs. Interestingly, despite the low stability, oligonucleotides containing the (phenylthio)methyl derivative **33** gave similar melting temperatures when paired with complementary strands with any of the four normal A, G, C and T bases opposite **33**. This suggests that it could be a candidate as a universal base, although further work must be carried out to verify this.

A different class of triazole-containing nucleobase analogue has been synthesised by Seela *et al.*[47] Toluoyl protected 1-azido-2-deoxyribofuranose **34** (Scheme 7) was reacted with a series of *N*-9-propargyl purines and *N*-1-propargyl-pyrimidines followed by treatment with sodium methoxide to give the unprotected nucleoside analogues which were studied as antiviral agents. The uracil and adenine analogues **35** and **36** were incorporated into oligonucleotides and their base pairing properties were studied. These A and T analogues were found to destabilise DNA duplexes. In another study of triazole-containing nucleobases a series of 5-(1,2,3-triazol-4-yl)-2′deoxyuridines were incorporated into DNA. In this case the modified bases stabilized DNA duplexes.[48]

The synthesis of extensively modified analogues of DNA in which the native phosphodiester linkages are replaced by triazoles throughout has been explored using alkyne nucleosides **38** and **40** with azide nucleosides **37** and **41**. Two types of triazole DNA backbones are described (**39** and **42**), differing in

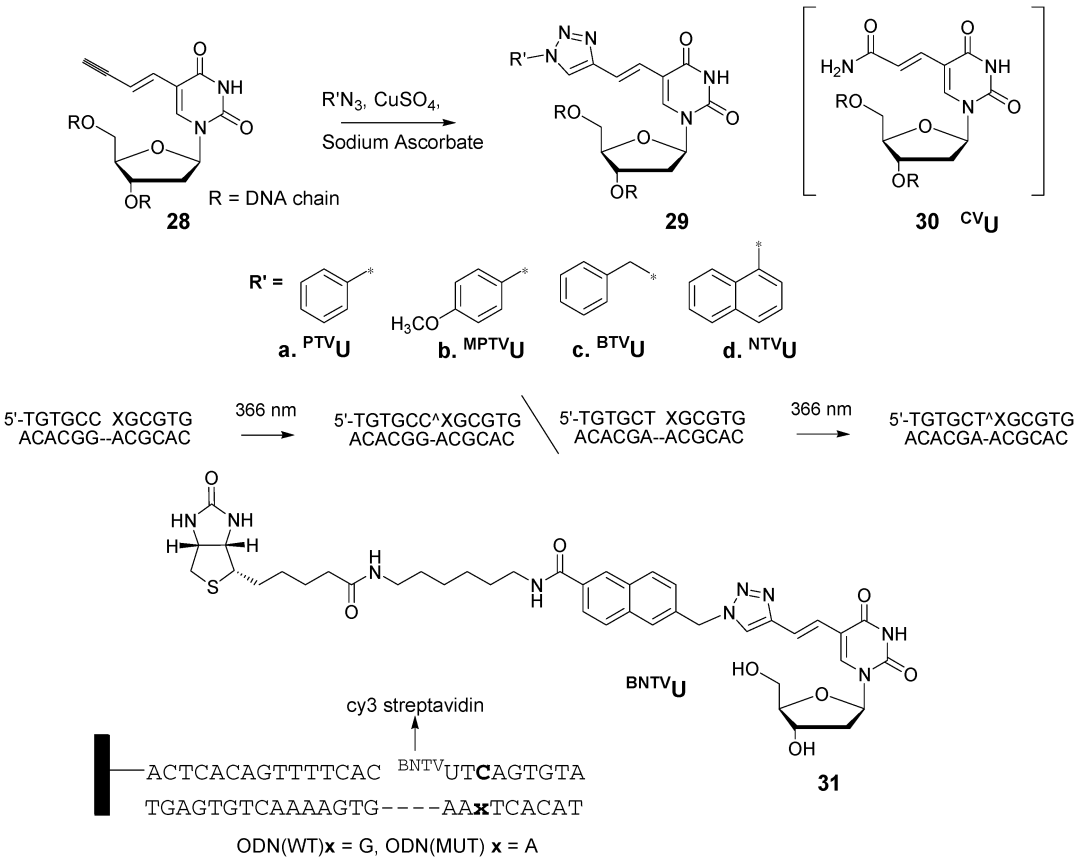
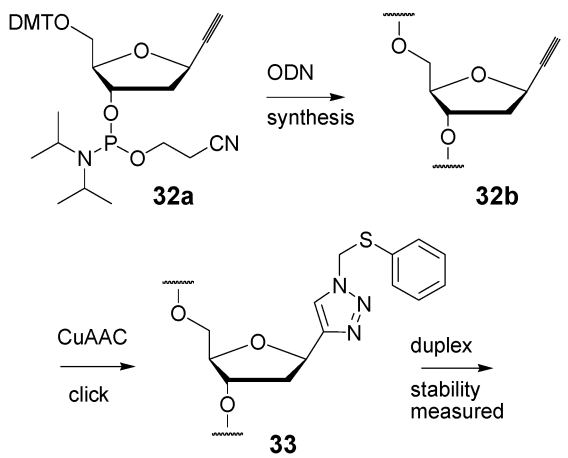
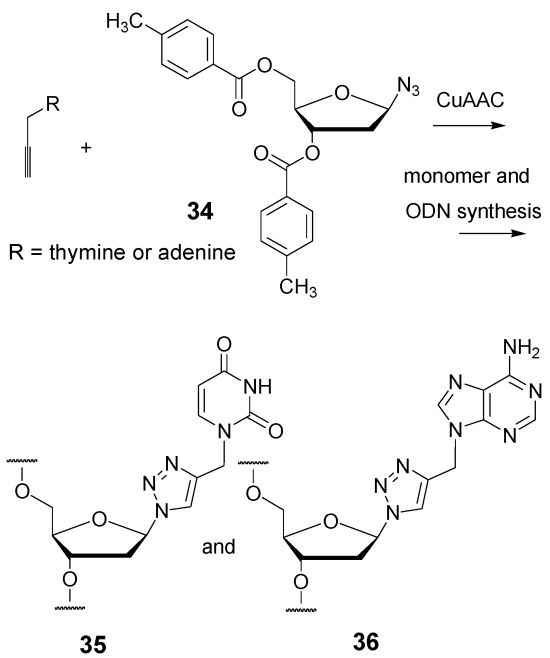


Fig. 9 DNA ligation and photo-cross-linking and its application to genetic analysis.



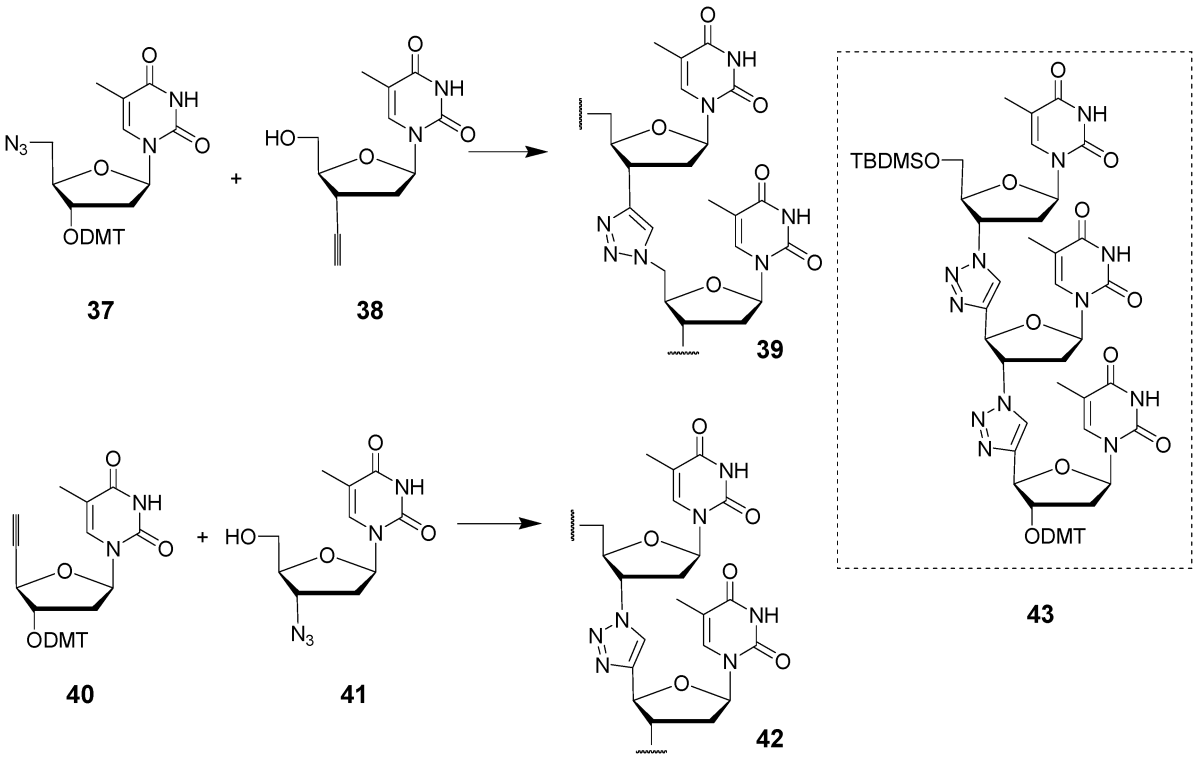
Scheme 6 Synthesis of DNA strands containing triazole base analogues.



Scheme 7 Synthesis of DNA containing triazole-bridged nucleobases.

the number of atoms in the unnatural backbone. A triazole dT_3 trimer **43** was synthesised as a model for longer oligomers (Scheme 8).[49]

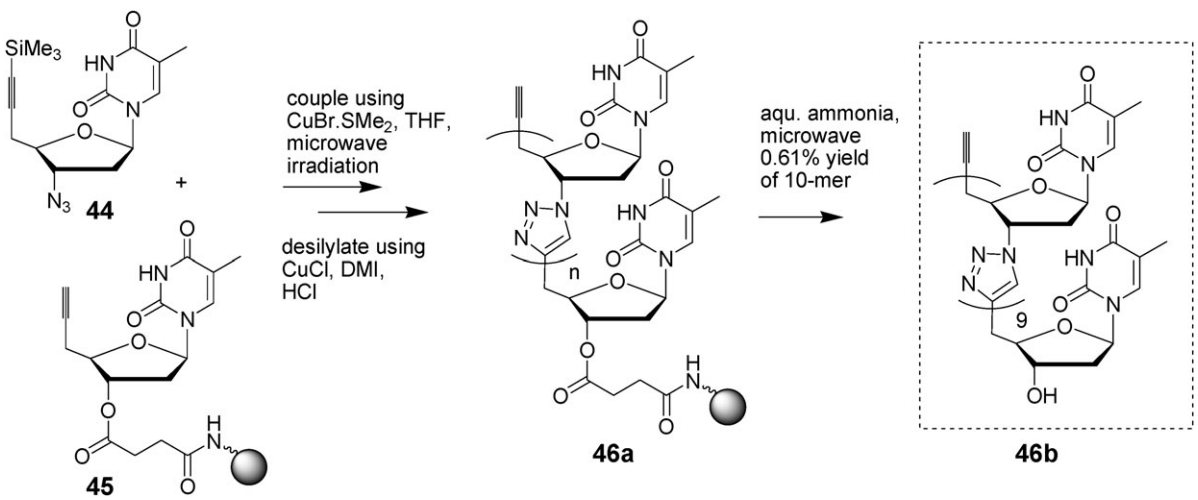
This idea was developed further by Isobe *et al.* who designed and synthesised a new triazole-linked analogue of DNA (^{TL}DNA)[50] using the CuAAC reaction (Scheme 9). This analogue differs from those synthesised by Dondoni *et al.*,[49] and the three analogues constitute an interesting set of DNA mimics. The analogue of DNA made by Isobe *et al.* utilizes a highly efficient and selective route that should be amenable to large-scale synthesis. Azide **44** and alkyne nucleoside **45** were reacted to give protected oligomers **46a** which on deprotection



Scheme 8 Synthesis of DNA analogues with unnatural triazole backbones (Dondoni *et al.*)[49]

yielded functional oligomer **46b**. The structural features of the new analogue, such as the rigid π-rich backbone and possibility of metal-coordination of the triazole rings may prove to be important in the structural chemistry and biology of oligonucleotides.[50]

The oligo-dT made by this method is soluble up to 7 μmolar in water and has greater solubility in the presence of acetonitrile. Solubility could be further increased by adding hydrophilic residues such as hexaethylene glycol at specific loci. Importantly the melting temperature (T_m) of the duplex with a normal DNA complement is 61 °C, which is much higher than the corresponding native $dA_{10}.dT_{10}$ DNA duplex (T_m = 20 °C). The authors suggest that the six-bond periodicity of the backbone is essential for duplex stability, quoting papers in which longer triazole backbones have been shown to destabilise the double strand. This promising analogue of DNA could compete with PNA as an analogue of DNA if the routine synthesis of mixed sequence ^{TL}DNA can be perfected. To this end, a convergent route for the solution-phase synthesis of ^{TL}DNA has recently been developed by the same authors. A one-pot procedure for desilylation of a masked acetylene and copper-catalyzed Huisgen coupling reaction between oligomers allowed the solution-phase synthesis of hepta- and octathymidine ^{TL}DNA in good yield.[51] Further development of ^{TL}DNA will require the synthesis of oligomers containing all four Watson–Crick nucleobases.



Scheme 9 Synthesis of an oligo-dT ^{TL}DNA analogue by Isobe *et al.*[50,51]

Oligonucleotide ligation, cyclisation and catenane formation

Oligonucleotide ligation

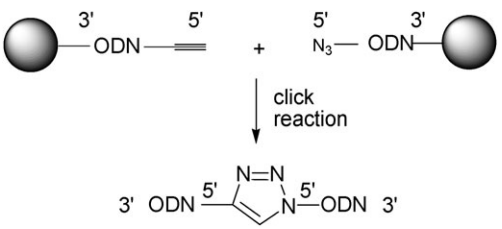
It is interesting to contemplate the use of click chemistry to ligate DNA strands, and subsequently amplify the ligated DNA by PCR. This would offer a method of increasing the utility of chemically modified oligonucleotides in molecular biology and genome analysis. There are currently no methods of chemically ligating DNA strands that work with high efficiency on a short timescale, and enzymatic methods that use ligases have several limitations, not least of which is the necessity to work on a small scale. If fast and efficient chemical ligation methods can be developed, the size range of synthetic oligonucleotide analogues available for gene synthesis and related applications will be greatly extended, provided that the resultant modified DNA backbone is a good substrate for enzymes used to replicate DNA. For PCR to be successful on such modified backbones, the triazole linkage must be a good mimic of normal DNA, and in an attempt to produce such a biologically compatible chemical linkage, a ligated DNA strand containing an unnatural T-triazole-T linkage has been synthesised by click ligation between an oligonucleotide with 3′-AZT **47** and another with 5′-propargylamido dT (**48)** (Scheme 10). The two oligonucleotides were held together by a complementary template oligonucleotide during the ligation reaction. The resulting click-ligated DNA strand (**49)** was used as a template in PCR, and amplification was successful with several different thermostable polymerases. DNA sequencing of the PCR amplicon and clones revealed the presence of a single thymine at the ligation site (**50)** instead of the two thymine bases that were present in the original template.[52] This is the first time that highly efficient non-enzymatic DNA strand ligation has been combined with reproducible amplification. One challenge in the above strategy was the synthesis of ODN **47**. To avoid the problems encountered by the instability of azides to P^{III} the DNA sequence up to the AZT was made by "reverse" solid-phase phosphoramidite oligonucleotide synthesis (5′- to 3′- synthesis) and then AZT was finally added as a phosphotriester monomer. Interestingly it has been shown that the instability of azides to P^{III} is only a problem if the phosphoramidite group and the azide are present in the same monomer, *i.e.* if an intramolecular Staudinger reaction can occur. Therefore, incorporation of azide monomers such as 4′-azidomethylthymidine can be carried out using an H-phosphonate monomer and the remainder of the oligonucleotide can be synthesised by conventional phosphoramidite chemistry.[53]

The remarkable ability of thermostable polymerases to copy DNA templates containing such an unnatural backbone opens up intriguing possibilities in gene synthesis, genetic analysis, biology and nanotechnology. In order to fully exploit this discovery it will be necessary to develop robust methods to synthesise long oligonucleotides bearing a suitable alkyne at one terminus and an azide at the other. This could enable the simultaneous template-mediated ligation of several ODNs to construct functional genes.

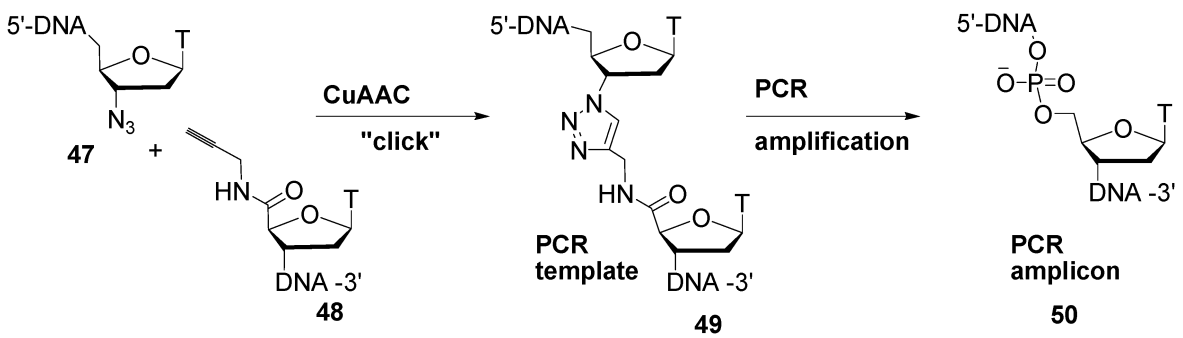
Chemical ligation of DNA strands (click ligation) has many potential uses, some of which are highlighted below, including 5′–5′and 5′–3′ strand ligation, the synthesis of cyclic DNA catenanes (synthetic mimics of mini-plasmids),[54] very stable mini-DNA duplexes,[55] and also duplexes that are covalently cross-linked across the nucleobases (discussed above).[41]

In a study to develop methods of attaching oligonucleotides to surfaces, solid phase non-templated chemical ligation of pairs of oligonucleotides by the CuAAC reaction has been used to produce oligonucleotides joined by 5′–5′ linkages (Scheme 11).[56] Such ODNs are of potential value in the triplex field.[57]

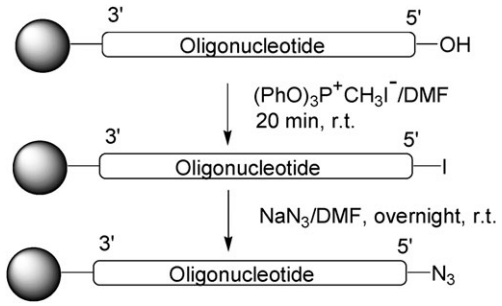
An essential part of this study was the development of methods for introducing functional groups at the 5′-end of oligonucleotides. This was achieved by reacting 5′-amino-oligonucleotides with compounds carrying the desired functional group linked to a carboxylic acid. The authors also used the phosphoramidite of 10-hydroxydecanoic acid *N*-hydroxysuccinimide ester (NHS ester) to introduce an amine-reactive NHS ester group at the 5′-end which was treated with propargylamine to give a 5′-alkyne functionalised oligonucleotide. In a simpler alkyne labelling protocol the



Scheme 11 The copper-catalyzed [3 + 2] cycloaddition between ODNs carrying azide and alkyne groups at their 5′ positions.



Scheme 10 Synthesis and PCR amplification of an unnatural triazole-based DNA backbone.



Scheme 12 Synthesis of oligonucleotides containing an azide group at the 5′-end *via* a 5′-iodo-oligonucleotide.

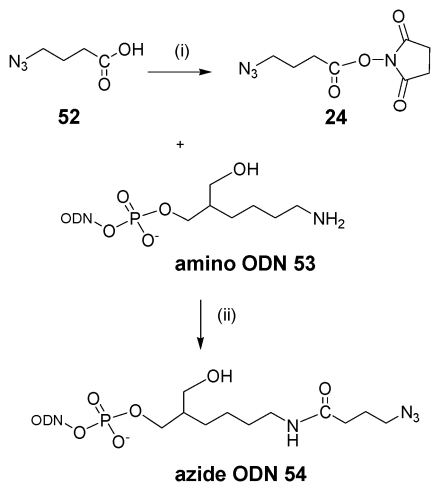
phosphoramidite derivative of commercially available hex-5-yn-1-ol was prepared and used to introduce the hexynyl group at the 5′-end of oligonucleotides. This "unactivated" alkyne was found to have poor reactivity in the CuAAC reaction. However, in a study of DNA cyclisation this alkyne was reported to give high yields of triazole-linked DNA.[55] For the synthesis of oligonucleotides carrying azide groups at the 5′ end, the authors used a method involving treatment of an amino-labelled oligonucleotide on the solid support with 5-azidopentanoic acid NHS ester. A second method involved replacement of the 5′-hydroxyl group of an oligonucleotide with iodine[58–60] which in turn was displaced by sodium azide to yield a 5′-azido-oligonucleotide (Scheme 12).[61] A related method involved addition of the phosphoramidite of 6-bromohexanol to the 5′-end of an oligonucleotide followed by displacement of bromine with azide.

The copper-catalyzed azide–alkyne cycloaddition reaction has been used for the template-mediated chemical ligation of two oligonucleotide strands, one with a 5′-alkyne and the other with a 3′-azide (Scheme 13) to produce a DNA strand with a continuous 5′- to 3′- backbone and a lengthy triazole linkage **51** at the ligation point.[54]

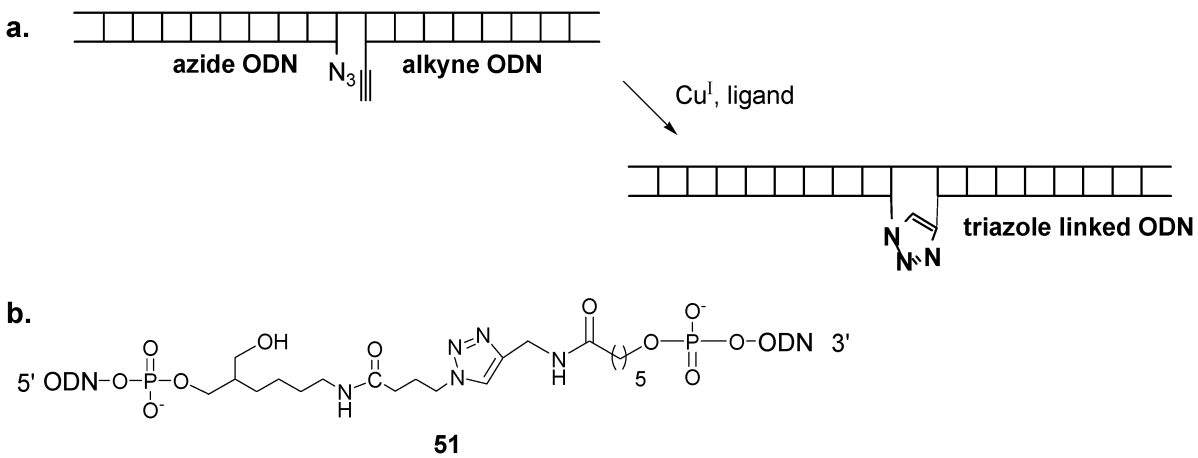
In order to achieve this, convenient syntheses of the relevant alkyne- and azide-modified oligonucleotides were required (Schemes 14 and 15). As azides are generally not stable in the presence of P^{III} they cannot be converted to functional phosphoramidite monomers for use in oligonucleotide synthesis. However, the active esters of organic azides can be added to aminoalkylated oligonucleotides in very high yields after solid-phase synthesis (post-synthetic modification). Using this strategy, azide-ODN **54** was prepared (Scheme 14) by reacting 3′- amino-C7-modified ODN **53** with 4-azidobutyric acid NHS ester **24** of carboxylic acid **52** in bicarbonate buffer at pH 8.75.

Alkyne ODN **60** was prepared using phosphoramidite monomer **58** in automated solid-phase oligonucleotide synthesis (Scheme 15). To prepare the required phosphoramidite monomer, the TBS ether of 6-hydroxycaproic acid **55** was converted to the corresponding propargylamide **56** followed by removal of the TBS protecting group to give alcohol **57**, phosphitylation of which gave phosphoramidite monomer **58**. This alkyne is mildly activated for the CuAAC reaction by the neighboring amide moiety. It was incorporated into the required ODN by standard solid-phase oligonucleotide synthesis to give support-bound ODN **59** which was cleaved and deprotected to give ODN **60**.

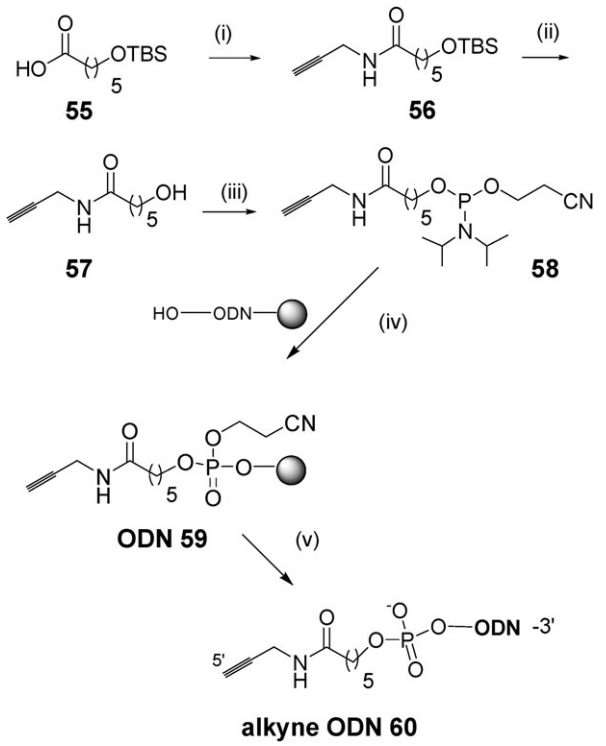
The Cu^{I} click catalyst was prepared *in situ* from aqueous Cu^{II} sulfate and sodium ascorbate, and all ligation reactions were carried out in 0.2 M NaCl to ensure duplex formation



Scheme 14 (i) NHS, DCC, DCM, 63%; (ii) 0.5 M $Na_2CO_3/NaHCO_3$ buffer (pH 8.75), DMSO.



Scheme 13 Template-mediated click-ligation of two oligonucleotides. (a). Schematic. (b). Chemical structure at ligation point.



Scheme 15 Synthesis of 5′-alkyne oligonucleotide. (i) Propargylamine, EDC, HOBt, DIPEA, DCM, 75%; (ii) TBAF–THF, 63%; (iii) 2-*O*-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite, DIPEA, DCM, 55%; (iv) oligonucleotide synthesis; (v) cleavage and deprotection with ammonia.

with the complementary template. The reaction of azide ODN **54** with alkyne ODN **60** proceeded efficiently in the presence of Cu^{I}-binding ligand HPTA **61**, to give the ligated ODN with backbone **51** at the point of ligation (Scheme 13). HPTA is a water soluble analogue of TBTA which is commonly used to accelerate the CuAAC reaction in organic solvents (Fig. 10).[13] At concentrations below 2.0 μM the ligation reaction did not proceed at a significant rate in the absence of the template oligonucleotide. This is interesting when contemplating simultaneous template-mediated ligation of multiple alkyne/azide-labelled DNA strands. It suggests that such an objective is achievable provided that the DNA concentration is sufficiently low to prevent undesirable non-templated reactions from occurring. In addition, the requirement for Cu^{I} catalysis of the ligation reaction means that oligonucleotide duplexes can be mixed and annealed to give the correct alignment of DNA strands before the click ligation reaction is switched on by addition of Cu^{I}. The uncatalysed reaction is extremely slow, so no unwanted reactions occur during the annealing process.

Melting experiments were carried out to investigate the effects of the triazole linkage on the duplex forming properties of the ligated oligonucleotide. The modified duplex displayed a lower melting temperature probably due to loss of base stacking caused by the extreme length of the extended unnatural backbone. The potential for varying the nature of this linkage to produce more stable duplexes simply by varying the chemical structures of the alkyne and azide has recently been explored.[52]

Oligonucleotide cyclisation

The click chemistry approach has been applied to the assembly of single-stranded DNA closed circular constructs using oligonucleotides labelled with a 5′-alkyne and 3′-azide. Such ODNs are straightforward to synthesise because 5′-alkyne oligonucleotide precursors containing 3′-amino functions can be efficiently labelled at the amine with azidoalkyl carboxylic acid NHS ester derivatives such as **24** (Scheme 14). Undesirable cycloaddition reactions do not occur between the 5′-alkyne ODN and the azide labelling reagent **24** because the AAC reaction is extremely slow in the absence of Cu^{I}. After oligonucleotide purification, non-templated click ligation was used to cyclise single strand oligonucleotides 12 and 72 bases in length using the alkyne and azide labelling procedure outlined above.[54] Quantitative cyclisation was achieved for the shorter constructs and the cyclic nature of the oligonucleotides was confirmed by HPLC, gel electrophoresis, mass spectrometry and restriction enzyme digestion of duplexes.

Formation of an oligonucleotide catenane

The above approach was applied to the assembly of a double-stranded DNA catenane of pseudo-hexagonal geometry with each side consisting of a single turn of B-DNA. To construct the catenane, two complementary oligonucleotides were labelled with a 3′-azide and 5′-alkyne as described above. Tandem T.T mismatches were placed after every 10 base pairs of Watson–Crick duplex to produce points of flexibility. Without these "hinges" such a short duplex would be too rigid and would be incapable of bending to form a complete circle. To form the catenane, the first ssDNA closed circle was prepared by an intramolecular click ligation reaction using Cu^{I}-catalysis. Template-mediated formation of a dsDNA pseudo-hexagon was then carried out by mixing the purified circular ssDNA with its linear complement (Fig. 11). After the click ligation reaction, a new retarded band appeared on the denaturing polyacrylamide gel owing to formation of the covalently closed double-stranded catenane.[54]

Modelling studies showed that a structure in which the strands are entwined six times (Fig. 12) can be formed, as well as constructs in which the single-strands have fewer crossovers.

In a recent study the synthesis of cyclic, branched, and bicyclic oligonucleotides has been performed by the microwave-assisted CuAAC reaction, both in solution and on solid support. New phosphoramidite building blocks and new solid supports were designed to introduce alkyne and bromo functions into the same oligonucleotide by solid-phase synthesis. The bromine atom was then substituted by sodium azide to yield azide oligonucleotides. Cyclisations were found to be more efficient in solution than on solid support. This method allowed the efficient preparation of cyclic (6- to 20-mers), branched (lariats with one or two dangling sequences), and bicyclic (2 × 10-mer) oligonucleotides.[62]

Cyclic mini-duplexes

The CuAAC reaction was also used to construct very stable cyclic DNA duplexes with as few as two base pairs.[55] The precursors to the cyclic duplexes were hairpin oligonucleotides

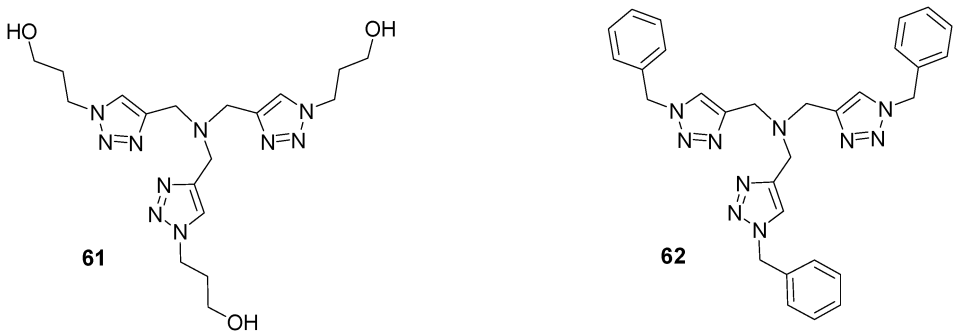


Fig. 10 Water soluble tris-hydroxypropyltriazolylamine Cu^{I}-binding ligand HPTA (**61**) and TBTA (**62**) which is useful for reactions in organic solvents.

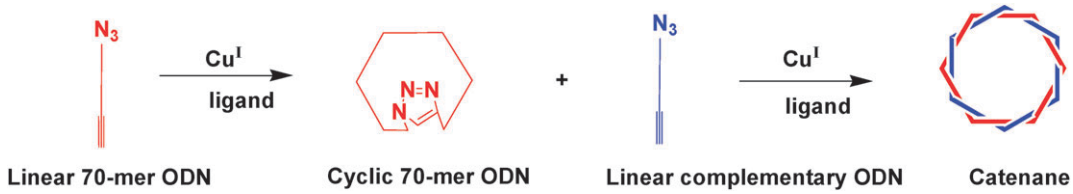


Fig. 11 Formation of double-stranded DNA catenane from single-stranded cyclic template ODN and linear complementary strand.

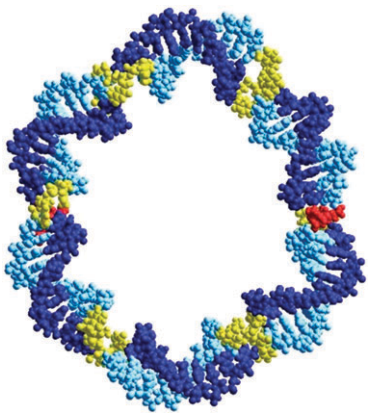


Fig. 12 Double stranded DNA catenane. Unpaired TpT/TpT hinge segments are shown in yellow, click-ligated regions in red and double-stranded regions in light and dark blue. In this representation of the catenane the two strands are entwined 6 times.

with a 5′-terminal alkyne, a 3′-azide and a loop region consisting of hexaethylene glycol units. Two different alkynes were used, the first based on 6-propargylamidohexanol phosphoramidite monomer **58** (Scheme 15), and the other, which is shorter by four atoms, was made by phosphitylation of 5-hexyn-1-ol. These monomers were incorporated at the 5′-end of hairpin oligonucleotides which were then labelled with 3′-azides and used in intramolecular self-templated click ligation reactions.

The CD spectra of the cyclic constructs showed the presence of helically stacked nucleotides, consistent with the B-family of conformations. In general these mini-duplexes are remarkably similar to normal double-stranded DNA. The one exception is the very high thermodynamic stability of the base pairs which makes them excellent model systems for detailed studies on DNA. This high stability should be particularly useful in cases when it is desirable to carry out high resolution investigations on modified DNA, and conventional duplexes are too unstable to permit this.

In general cyclic duplexes have very high stability,[55] and the length of the linker at each end has a significant influence on the degree of stabilisation. The dependence of duplex stability on length of linker has been observed previously with hairpin oligonucleotides.[63] The precise length of the triazole linker formed in the click cyclisation reaction also influences the stability of cyclic duplexes. Combining the most stable linkages produced a two base pair cyclic duplex with a 19 atom HEG linker and 20 atom triazole linker which formed a remarkably stable two base-pair duplex, the T_m of which was above the measurable range in aqueous buffer. ^{1}H NMR studies in H_2O clearly showed the H-bond-mediated G.C base pairing. A dinucleotide duplex is the shortest in which base stacking is possible. Its stability is due to a combination of electrostatic and aromatic interactions, plus inter-base hydrogen bonding. A cyclic GC construct with a single base in each strand (C-1) did not show any evidence of base pairing, presumably because there is no possibility of duplex stabilisation by base stacking. The interaction of these cyclic mini-duplexes with DNA-binding drugs is sequence-dependent, as would be predicted from equivalent linear constructs, confirming that they are essentially "normal" DNA duplexes. In a subsequent study on DNA drug binding, the mode of action of a novel threading intercalator was elucidated using an end-sealed duplex that was cyclised using the CuAAC reaction.[64] As oligonucleotide degradation is rapid in the presence of free cuprous ions,[14] the effect of reducing the quantity of Cu^{I} on the efficiency of the CuAAC oligonucleotide cyclisation reaction was also investigated. As little as 1.25 equiv. of Cu^{I} was sufficient for efficient click ligation of short oligonucleotides.[65]

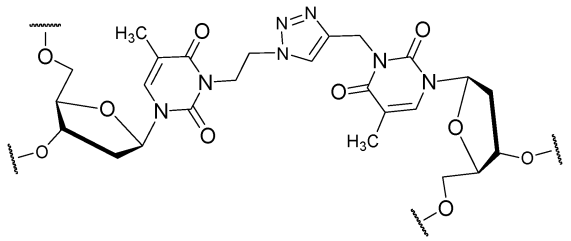


Fig. 13 Linkages between the N3 atoms of thymine bases used to construct cyclic dumbbell oligonucleotides.

In order to assess the stability of cyclic duplexes in biological media, a series of hairpin oligonucleotides were cyclised using the CuAAC reaction. The resultant cyclic constructs were designed as decoys for targeting the DNA binding site of the TCF/LEF and GLI transcription factors.[66] Incubation of a 20 base pair fluorescein-labelled cyclic oligonucleotide and its hairpin counterpart in fetal calf serum showed that the cyclic construct has significantly greater stability to enzymatic degradation. Cell uptake studies on the cyclic construct using wt HEK-293 cells in the presence of Lipofectamine 2000 transfection agent demonstrated that the ODN is taken up by the cells, and localizes to the nucleus after only 1.5 h. Intranuclear fluorescence increased over a period of 4 h and persisted for 24 h.

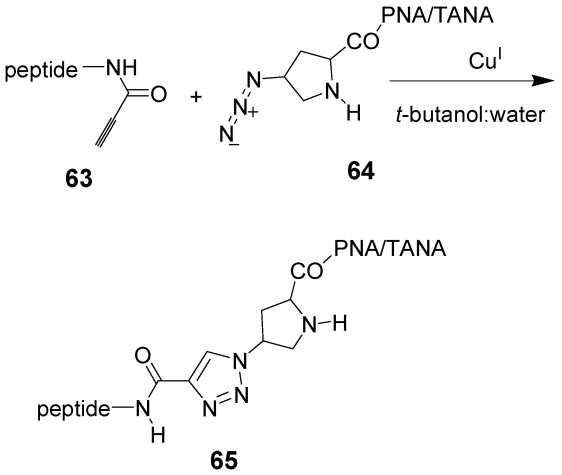
Cyclic dumbbell oligonucleotides have been synthesised and shown to have high melting temperatures and good stability to snake venom phosphodiesterase (3′-exonucleoase). Such oligonucleotides have been shown to bind to NF-κB p50 homodimer, and have potential use as *in vivo* decoys for important regulatory proteins. These dumbbells had oligo-dT loops at the ends, and it was found that the greater the number of thymines, the better the protein binding.[67] The click linkages joining the ends of the cyclic dumbbell were made between the N3 atoms of thymine bases (Fig. 13).

Conjugation of peptides to DNA and PNA

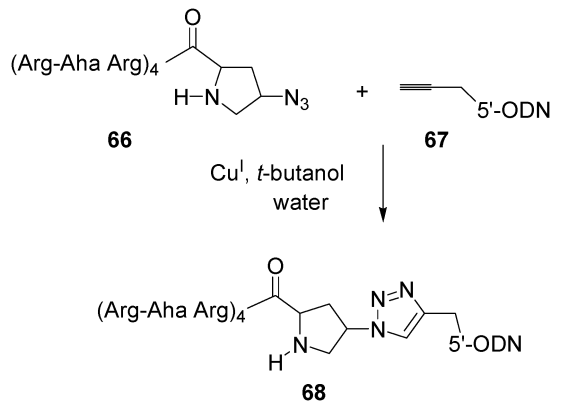
Peptides have been conjugated to PNA and to other oligonucleotides analogues such as thioacetamido nucleic acids (TANA) and also to normal oligonucleotides by the CuAAC reaction.[68] The transformations were carried out by reacting alkyne derivatised peptides **63** with azide labelled PNA or TANA **64** to produce conjugates **65** (Scheme 16), or azide peptide **66** with alkyne labelled DNA **67** to give oligonucleotide–peptide conjugate **68** (Scheme 17).

Nucleic acid triplexes and quadruplexes

Click chemistry has recently been used to produce modified triplex forming oligonucleotides (TFOs). For this purpose, an efficient method was developed for post-synthetic modification of oligonucleotides incorporating internal insertions of (*R*)-1-*O*-(4-ethynylbenzyl)glycerol through the microwave assisted CuAAC reaction with pyren-1-yl azide. The twisted intercalating triplex forming nucleic acids (TINA TFOs) obtained in these reactions possessed site-specific insertions of (*R*)-3-*O*-4-[1-(pyren-1-yl)-1*H*-1,2,3-triazol-4-yl]benzylglycerol. These pyrene-modified oligonucleotides displayed high affinity for their duplex targets.[69]



Scheme 16 Click ligation of alkyne-functionalised peptide with 4-azidoprolyl labelled PNA/TANA.



Scheme 17 Click ligation of DNA-alkyne with 4-azidoprolyl-peptide.

Four-stranded DNA structures formed by guanine-rich sequences (G-quadruplexes) have received much attention owing to their remarkable structural properties and biological importance. G-quadruplex DNA plays an important role in telomere maintenance and is a potential tumor-selective target for chemotherapy. A G-quadruplex can act as a scaffold for a CuAAC reaction and the reaction of a 5′-alkyne with a 5′-azide, of a 3′-alkyne with a 3′-azide, and of a 5′-alkyne with a 3′-azide can occur in different types of G-quadruplex structures.[70] This method has been used to probe the structures of G-quadruplexes, the most important finding being that a DNA–RNA hybrid G-quadruplex structure can be formed from human telomeric DNA and RNA sequences. The isolation of "all-DNA" and "all-RNA" quadruplexes was avoided by using alkyne-labelled RNA and azide-labelled DNA in the reaction. The advantage of the click reaction in this context is that it can trap a particular species, or produce a snapshot of the various inter-converting structures that are present in a complex solution, whereas traditional methods such as NMR spectroscopy, X-ray crystallography and FRET studies are instead more likely to identify averaged structures and the most abundant components.

This review focuses on the CuAAC reaction, but there are other viable click reactions that can be used in DNA

chemistry, including the Staudinger ligation.[8] This reaction has been used extensively in various biological systems,[71–73] and has been employed to label DNA.[74,75] Applications of the Staudinger reaction in its "traceless" form are particularly interesting.[76]

Summary

There are probably no clearer examples of the extraordinary power and versatility of the CuAAC reaction than those found in the field of nucleic acids chemistry. Although the CuAAC reaction was only recently discovered[1,2] and references to DNA click chemistry essentially date back to just 2006, there have been many important and useful developments in DNA labelling. The ability of DNA strands to hybridize in a predictable and controlled manner to provide a template for the reaction of alkynes with azides has facilitated the synthesis of cyclic DNA strands and other structures of potential value in biology and nanotechnology. Entirely artificial DNA analogues are beginning to emerge, but so far *in vivo* applications with DNA have been slower in coming to fruition. This is in part due to the toxicity of Cu^{I} to cells, making the CuAAC reaction apparently inapplicable, but also to the greater challenges associated with chemical intervention in biological systems. However, important work in this area is beginning to emerge employing both copper-catalysed[77] and uncatalysed versions[78] of the AAC reaction.

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