Click Nucleic Acid Ligation: Applications in Biology and Nanotechnology

AFAF H. EL-SAGHEER†,‡ AND TOM BROWN*†

†School of Chemistry, University of Southampton, Highfield, Southampton SO17 1BJ, United Kingdom, and ‡Chemistry Branch, Department of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez Canal University, Suez 43721, Egypt

CONSPECTUS

Introduction

Current biochemical strategies to produce large DNA constructs utilize a combination of synthetic oligonucleotides, thermostable DNA polymerases, and DNA ligases. Entire bacterial and mitochondrial genomes and sections of eukaryotic chromosomes up to 1 megabase in length can be assembled using such approaches.1–3 Such ambitious targets are feasible because the error rates of proofreading...
DNA polymerases are lower than $10^{-6}$. In comparison, technologies for the chemical synthesis of long DNA strands lag far behind. The best available chemical approach is the solid-phase phosphoramidite method, which can be used to assemble DNA strands up to 150 bases in length. Beyond this point, deficiencies in the chemistry make it impossible to produce pure DNA. This is in part due to the physical properties of the resins on which the synthesis is conducted, but it is also a consequence of imperfect coupling and undesirable side-reactions that create mutagenic modifications to the nucleobases. For very long oligonucleotides, such modifications cannot be removed by purification, and error rates as high as $10^{-2}$ have been reported. A possible alternative approach to the chemical synthesis of large DNA strands is to join together carefully purified synthetic oligonucleotides by chemical methods. However, it has proved challenging to achieve clean and efficient chemical ligation of oligonucleotides, although progress has been made using cyanogen bromide as a coupling agent. An alternative strategy is to design a chemical linker that mimics the natural phosphodiester bond and which can be formed in high yield in aqueous media from functional groups that are orthogonal to those present in DNA. This goal has been partly achieved by the reaction between oligonucleotides with $3'$-phosphorthioate and $5'$-tosylate or iodide. Unfortunately, this chemistry does not satisfy the key requirements of a robust chemical method of gene synthesis, namely, the use of functional groups that are stable in aqueous media, the ability to synthesize bifunctional oligonucleotides with reactive groups at each end for use in multiple ligation reactions, a mechanism to initiate the ligation reaction when participating oligonucleotides have been hybridized to complementary splints (to arrange the DNA strands in the desired order by templated preassembly), and the creation of a very stable backbone linkage.

If an efficient chemical ligation method was available, it would increase the maximum attainable size of chemically synthesized DNA, and might even be applicable to the total chemical synthesis of genes. Such a method could gain popularity in biotechnology and nanotechnology where the availability of large quantities of very long chemically modified oligonucleotides would facilitate experiments which have hitherto been impossible to perform. However, its utility in biotechnology will only be fully realized if DNA strands containing the artificial linkages are able to act as templates in DNA replication. This is a tall order; the phosphodiester linkage is ubiquitous in the DNA of living systems, and there is no reason a priori to expect DNA polymerases to tolerate an artificial backbone. However, polymerases do have the capacity to read through damaged DNA, and engineered enzymes can perform this feat with surprising efficiency. These developments provided an impetus for the design of a biocompatible DNA backbone linkage.

**Design of a Biocompatible Click DNA Linkage**

In order to produce a biocompatible chemical linkage, we turned to click chemistry, a concept that was developed to join together organic molecules under mild conditions in the presence of a diverse range of functional groups. The best example of click chemistry is the Cu-catalyzed version of Huisgen's [3 + 2] azide–alkyne cycloaddition reaction, recently discovered independently by Sharpless and Meldal (the CuAAC reaction). This highly selective and efficient reaction was chosen for nucleic acid ligation for several reasons; alkynes and azides can be attached to nucleic acids by simple methods, the CuAAC reaction proceeds efficiently in aqueous media, the reaction can be switched on at will by addition of Cu, and the resultant triazole unit is extremely stable. Studies in the fields of reaction discovery, templated synthesis, and nanotechnology had shown that this chemistry is applicable to DNA, and the new challenge was to design a triazole linkage with a structure that is biocompatible. Initial investigations involved the synthesis of DNA containing the unnatural triazole linkage A (Figure 1d). This is formed by the reaction between oligonucleotides functionalized with $3'$-azido-dT and $5'$-propargylamido-dT in the presence of a complementary template oligonucleotide (splint) which holds the reactants in close proximity. The click reaction also occurs in the absence of the splint, but it is much slower and requires higher oligonucleotide concentrations. Splints confer another important advantage; they allow the assembly of multiple alkyne/azide DNA fragments in the correct order prior to Cu-catalysis. A click-ligated DNA strand containing such a triazole linkage was used as a template in PCR (Figure 1a). Amplification was successful but DNA sequencing of the progeny strands revealed only a single thymidine at the ligation site instead of the two thymidines of the original template. Although this was disappointing the overall result was encouraging. Importantly, it provided the first example of efficient chemical DNA strand ligation combined with PCR amplification through an unnatural DNA backbone linkage. It also gave hope that a truly biocompatible DNA backbone linkage could be designed if the limitations of the first
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With these considerations in mind, a more flexible second generation triazole linkage was synthesized (linkage B, Figure 1d).

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FIGURE 1. (a) Synthesis and PCR amplification from the first generation triazole DNA backbone linkage. (b) cis–trans isomerization of amide bond. (c) Replication bypasses one of the thymine bases around the triazole linkage. (d) First generation triazole linkage A, second generation triazole linkage B, triazole linkage C in TL-DNA, and canonical DNA linkage D. The number of chemical bonds between the C3' and C4' atoms of adjacent sugar rings is written in blue.
through accurately. Successful linear enzymatic copying of one of the 81-mers was then carried out to prove that PCR amplification of the chemically modified DNA was not a rare event. To demonstrate the utility of click chemistry for the assembly of large DNA molecules with more than one click ligation site, three 100-mer oligonucleotides were ligated to give a 300-mer PCR template with two triazole linkages. This template was subjected to PCR and sequenced, and the amplicon was shown to be a faithful copy of the original template. The synthesis of cyclic DNA was also achieved; a 100-mer oligonucleotide with 5'-azide and 3'-alkyne functionalities was cyclized by addition of CuI to produce a construct containing a single triazole (linkage B, Figure 1). Rolling circle amplification (RCA) was then carried out to produce a linear construct containing multiple copies of the original cyclic template (Figure 2).

**In Vivo Biocompatibility of Triazole Linkage B**

The biocompatibility of the modified DNA was investigated in *E. coli* using a plasmid containing a triazole linkage in each strand of its antibiotic marker gene (Figure 3). The number of colonies obtained from *E. coli* transfected with the modified plasmid was similar to the native plasmid, and DNA sequencing confirmed that the bases around the triazole linkage were copied correctly. The experiments were repeated in a strain of *E. coli* deficient in UvrB, an essential component of DNA repair. Transformation with the triazole plasmid gave 93% of the number of colonies from the native plasmid, and the DNA sequence around the triazole was replicated accurately. This supports the hypothesis that DNA repair does not make a significant contribution to the biocompatibility of the artificial DNA backbone, and that replication occurs through the triazole linkage in vivo. A rationale for the surprising discovery that polymerases can copy a DNA template containing a completely unnatural backbone linkage comes from the X-ray structure of the complex between Taq polymerase and DNA. In this structure, several nucleotides in the DNA template interact with the enzyme. In the click-ligated DNA template, only one of the internucleotide linkages can be a triazole because the template is synthesized by click ligation of long oligonucleotides, and the triazole linkages only occur at the ligation points. Hence, very few hydrogen bonding interactions between the DNA backbone and the enzyme can be destroyed by the triazole substitution. This partly explains why triazole linkage B is read through correctly during PCR amplification. Further support comes from an NMR study of a DNA duplex containing a single triazole linkage. The triazole introduces local structural changes but leaves the duplex and Watson–Crick base pairs intact. Moreover, the N3 atom of the triazole ring can mimic the hydrogen bond acceptor properties of the phosphodiester group in a complex with DNA polymerase. It lies close to the position of a phosphate branching oxygen (Figure 4), and it has a large dipole moment and proven hydrogen bond acceptor capacity.

**Biophysical Studies on Triazole DNA**

The effect of the triazole linkage on the kinetics and thermodynamics of duplex formation has been explored by thermal denaturation studies on a 13-mer DNA duplex containing a single central triazole linkage. Both triazole and normal control duplexes gave sharp cooperative melting transitions (at 55 and 63 °C, respectively) with a similar degree of hyperchromicity, indicating that the triazole duplex is fully base paired. The effect of a mismatched base pair at the triazole site is particularly interesting. It lowers the
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duplex melting temperature by 11 °C on average, similar to a mismatch in the native duplex. The average decrease in hyperchromicity caused by a mismatch was 18.8% for the triazole duplex but only 7.5% for the native duplex, indicating that the combination of a triazole linkage and mismatched base pair causes a much greater loss of base stacking than is observed in native mismatched DNA. A detailed NMR study revealed that the opening rates of the TA base pairs on either side of the modified backbone are reduced by 11 kJ/mol compared to the native duplex, and that the destabilization is spread over the four base pairs immediately surrounding the triazole linkage. The Gibbs energy required to dissociate each of the two base pairs on either side of the triazole linkage has been shown to produce more destabilization of the DNA duplex but only 7.5% for the native duplex, indicating why it destabilizes the DNA duplex. Reducing the length of the triazole modification is perturbed rather more than the base pair on the 3'-side (7.2 and 3.4 kJ/mol, respectively). Despite this destabilization, the duplex accommodates the unnatural linkage in a normal B-DNA helical structure with little distortion of the major and minor grooves. The above biophysical data allow us to suggest a model of replication through the triazole linkage. When the correct dNTP is selected by the enzyme—polymerase complex, a normal Watson–Crick base pair forms as the incoming nucleotide is incorporated. In contrast, on the rare occasion that a mismatched dNTP is inserted, severe duplex instability and local melting triggers removal of the rogue nucleotide by the polymerase enzyme. This “double hit” of triazole linkage and mismatched base pair is an easy target for the proofreading functionality of DNA polymerase.

There are five bonds between the C3' and C4' atoms of adjacent sugar rings in a normal DNA backbone. In contrast, triazole linkage B is seven bonds in length, possibly explaining why it destabilizes the DNA duplex. Reducing the length of the triazole linkage has been shown to produce more stable duplexes; triazole linkage C (Figure 1) with five bonds has been used to construct T7 DNA in which every phosphate linkage is replaced by a triazole. It is important to state that although oligo dT T7 DNA forms a stable duplex with oligo dA,28 the properties of a single triazole linkage C in a normal DNA duplex have not yet been explored; its effects on duplex stability and its compatibility with DNA and RNA polymerases should be investigated.

Transcription through the Triazole Linkage
The biocompatibility of triazole-linked DNA in E. coli cannot be directly ascribed to efficient transcription through the modified backbone.20 This is because replication might have occurred prior to transcription, in which case the cellular RNA polymerase enzymes would not have encountered the artificial linkage. Therefore, to evaluate the suitability of triazole DNA for in vitro synthesis of RNA, it was necessary to carry out experiments in a controlled environment. This was done using T7 RNA polymerase (T7-RNAP), an enzyme which is used in biotechnology to direct the synthesis of small RNAs and proteins. The RNA transcript selected for this study contained the 54-mer DcF sequence29 which inhibits the growth of E. coli. Two triazole-containing DNA template strands were studied, one with the triazole linkage inside the coding sequence and a second with the modification within the essential T7 RNA promoter region. When the triazole linkage was placed in the coding region (Figure 5), the transcription reaction produced ~80% of the quantity of RNA obtained from the native control DNA template, and mass spectrometry showed that the two transcripts were identical.30 This demonstrates that T7-RNAP can accurately transcribe through triazole-modified DNA to synthesize fully complementary RNA in good yield. This is the first example of transcription through a purely synthetic analogue of a DNA backbone, and it suggests that DNA constructs made by solid-phase synthesis and click ligation can be used directly for protein expression. When the transcription reactions were repeated with the triazole in the promoter region of the DNA template there was no RNA product. This could be due to a lack of binding between the T7-RNAP and the triazole DNA. Further studies are required to confirm this.

Click Ligation for the Synthesis of RNA
In vitro transcription is commonly used to make long RNA strands, but this enzymatic process does not permit the site-specific incorporation of chemical modifications at sugars, bases, or phosphates. In contrast, the chemical synthesis of RNA by solid-phase methods offers advantages over biochemical approaches. It is compatible with the insertion of fluorescent tags, isotopic NMR labels, and modifications...
designed to improve the biological activity and stability of RNA. In addition, the scale of chemical synthesis is essentially unlimited. An efficient methodology for chemical ligation would be of great value as it would bring synthetic RNA into the size range required for many biological studies. Enzymatic ligation is an established method of synthesizing large RNA constructs from smaller fragments, but it cannot be used in certain situations, for example, to link together RNA strands across the bases or between modified sugars. Click ligation can overcome these limitations, and it has recently been used to synthesize the chemically modified hairpin and hammerhead ribozymes described below.

Synthesis and Properties of the Click Hairpin Ribozyme and Analogues

The hairpin ribozyme belongs to a family of catalytic RNAs that cleave their RNA substrates to generate 2′,3′-cyclic phosphate and 5′-hydroxyl termini. It was chosen for click ligation studies because its direct chemical synthesis is very difficult, its biochemical properties are well understood, and its functionality can be extended by the incorporation of modifications such as fluorescent tags. Hairpin ribozyme analogues were synthesized by cross-linking the side-chains of modified uracil bases in opposite strands of three separate RNA segments (trans-ligation). The individual oligonucleotides were prepared by standard solid-phase synthesis, and they were functionalized with an alkyne or azide group at the 5-position of selected uracil bases (Figure 6). To construct the ribozymes, the individual segments were linked in two simultaneous self-templated CuAAC reactions across the major groove of the ribozyme stems. Cross-strand linking between nucleobases in RNA is unique to chemical ligation and cannot be achieved by enzymatic methods. The catalytic activity of the click hairpin analogues was shown to be similar to the native ribozyme. The click ribozymes could be isolated from the gels of the cleavage reactions and reused with no significant loss of activity, demonstrating their high stability.

The Hammerhead Ribozyme

In an alternative approach to click RNA ligation, the hammerhead ribozyme, a small naturally occurring catalytic RNA, was made from two RNA strands by splint-mediated intrastrand click ligation (cis-ligation) of a 3′-alkyne and a 5′-azide oligoribonucleotide (Figure 7). This strategy produced a ribozyme containing triazole linkage B (Figure 1). The click hammerhead ribozyme cleaved its substrate with similar efficiency and specificity to the native ribozyme, confirming the biocompatibility of the triazole linkage, even though the unnatural triazole linkage is located at the active site between residues C3 and U4 of the ribozyme (Figure 7).

In a separate study, a combination of chemical and enzymatic synthesis was used to incorporate an azide group into RNA. Templated and nontemplated CuAAC ligation reactions were carried out between azide and alkyne-functionalized RNA. This provided active ribozymes containing a single triazole, confirming that the triazole backbone is not detrimental to RNA function. This is despite the fact that this artificial linkage destabilizes RNA duplexes, as shown in a UV melting and CD study on several short self-complementary...
RNA duplexes containing triazole linkages. NMR studies on an 8-mer duplex in which the triazoles were present in both strands and directly opposite to each other indicated that all base pairs were formed despite instability around the modified linkages. Cyclic Mini-DNA Duplexes
The CuAAC reaction has been used to construct very stable cyclic mini-DNA duplexes from hairpin oligonucleotides functionalized with a 5'-alkyne, a 3'-azide, and a hexaethylene glycol loop. They have similar properties to normal double-stranded DNA, a notable exception being their very high thermodynamic stability, which makes them excellent model systems for detailed studies on base pairing. 1H NMR studies on a cyclic GC/GC dinucleotide duplex (Figure 8) showed stable hydrogen bonding between the GC base pairs, and UV melting indicated that the caging effect holds the structure together, making the duplexes highly resistant to thermal denaturation. In contrast to the GC/GC dinucleotide, a cyclic construct with a single G in one strand and C in the other (a single base pair), showed no evidence of base pairing, presumably because there is no possibility of base stacking interactions. This illustrates the crucial influence of base stacking on DNA duplex stability. Cyclic duplexes are resistant to enzymatic degradation in biological media, and have potential uses as decoys for transcription factors. Incubation of a fluorescein-labeled 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 in the presence of a transfection agent showed that the cyclic oligonucleotide localized to the nucleus after only 1.5 h and persisted for 24 h. Other cyclic dumbbell oligonucleotides synthesized by click chemistry have also been shown to have high duplex melting temperatures and strong resistance to degradation by snake venom phosphodiesterase. These oligonucleotides were designed to bind to the NF-κB p50 homodimer as in vivo decoys. In a study on DNA drug binding, the mode of action of a novel threading intercalator was elucidated using a click-ligated cyclic duplex.

Synthesis of a DNA Catenane
Click chemistry has been applied to the assembly of a double-stranded DNA catenane consisting of six turns of B-DNA. To construct the catenane, a stepwise strategy was adopted as shown in Figure 9. Two complementary oligonucleotides were used, each labeled with a 3'-azide and a 5'-alkyne. One of them was cyclized in a nontemplated CuAAC reaction and used as a template for the second click cyclization. Tandem TT mismatches were placed at intervals of 10 base pairs in the cyclic duplex to produce points of flexibility, without which the duplex would be too rigid to bend into a circle.

Nanotechnology and Higher Order Structures
Controlled self-assembly makes DNA an ideal material for building nanostructures. However, an obstacle to its use in constructing intermediates in hierarchic assembly is the thermodynamic instability of the assembled double stranded nanoconstructs, which makes purification difficult. A click-fixation technology has been developed by which robust chemically cross-linked DNA nanoconstructs can be made and purified by denaturing gel-electrophoresis. Such chemically fused building blocks could be used to build larger nanonetworks. Triplex DNA binders have been used in...
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nanotechnology applications involving click chemistry. They can act as external stimuli for controlling chemical reactivity in higher-order DNA-directed reactions. Efficient double click reactions can be controlled simply by adding a strong triplex DNA binder such as naphthylquinoline. This is a high-yielding method for the formation of three-way branched nonsymmetrical DNA sequences that are useful in the construction of DNA nanostructures. Other higher order nucleic acid structures have been isolated in a stable form by click chemistry. Conformational isomers of G-quadruplexes have been trapped by performing click reactions between their termini. This approach led to the discovery of a DNA–RNA hybrid-type G-quadruplex derived from the sequence of the human telomere. The click fixation allows detection and identification of the G-quadruplex reaction products in a complex solution, whereas traditional methods such as NMR spectroscopy and X-ray crystallography were not suitable due to exchange between different quadruplex conformations.

A simple and efficient template-free technique for interstrand cross-linking of DNA has been developed using a “bis-click” CuAAC reaction between alkyne-functionalized oligonucleotides and bifunctional azides. The methodology is independent of base composition and is applicable to single stranded DNA, duplexes, and multistranded structures. Four-stranded DNA structures consisting of two cross-linked duplexes were obtained after hybridization of the bis-click constructs. This methodology has applications in nanobiotechnology and material science. The CuAAC reaction has also been used to ligate artificial mimics of DNA. A highly efficient chemical ligation method has been developed for quantitative conjugation of PNA with DNA or PNA with PNA. In this work, PNA oligomers with an alkyne at the C-terminus and an azide at the N-terminus were used in DNA-templated ligation reactions. This enabled the preparation of extended PNA sequences which are difficult to prepare by standard solid-phase methods. Templated PNA click ligation is sequence specific and capable of single nucleotide discrimination.

Copper-Free Click DNA Ligation

The reaction between azides and unactivated terminal alkynes is very slow in the absence of Cu catalysis, even when templated by DNA. However, the copper-free AAC reaction can be accelerated by using an alkyne which is distorted by incorporation into an 8-membered ring structure. The ring-strain promoted alkyne–azide cycloaddition reaction (SPAAC reaction) has been pioneered by Bertozzi and Agard, principally for in vivo applications involving carbohydrates. It has recently been applied to DNA strand ligation using dibenzocyclooctyne (DIBO) as the strained alkyne (Figure 10). Unlike electron deficient terminal alkynes, DIBO is stable in aqueous buffers and survives the conditions of oligonucleotide deprotection. The DNA-templated SPAAC ligation reaction is very fast (∼1 min at ambient temperature), and the presence of a single mismatched base pair is sufficient to stop the reaction, suggesting applications in genetic analysis. It should be possible to use this chemistry for multiple simultaneous templated DNA ligation reactions if participating oligonucleotides are labeled with either two alkynes or two azides.

The SPAAC reaction on DNA has advantages and disadvantages when compared to the CuAAC reaction. As it does not require catalysis with toxic metals, it has potential uses in vivo. However, undesired ligation reactions can occur before the participating oligonucleotides have had time to assemble against the template. At low reactant concentrations, the reaction does not proceed at an appreciable rate in the absence of DNA templating, so this may not be a major problem. The SPAAC reaction is orthogonal to the Diels–Alder reaction.
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which has also been used for DNA strand ligation.\(^5^5\) Unlike the CuAAC reaction, neither the SPAAC or the Diels–Alder reaction is likely to give rise to a DNA linkage that can be read through accurately by polymerase enzymes.

Conclusions and Outlook

Click chemistry on nucleic acids is clean, efficient, tolerant of most other functional groups, and compatible with large scale synthesis. Very long DNA and RNA strands have already been synthesized using this methodology, and the limits have not yet been fully explored. Careful design has given rise to a biocompatible triazole linkage that is treated as normal by DNA and RNA polymerases, and a gene containing triazole linkages has been shown to function in E. coli. These surprising results could lead to important future applications in biology and nanotechnology. For example, one could contemplate the use of click DNA ligation for the assembly of synthetic genes on a large scale; for site-specific incorporation of unusual base or sugar modifications (e.g., mutation hot spots, epigenic modifications) to study DNA replication, repair, and gene regulation, or to incorporate chemical modifications such as fluorescent dyes for in vivo imaging. In the RNA world, segmental labeling followed by the assembly of large chemically modified RNA constructs for functional studies is a distinct possibility. In addition, a number of therapeutic applications could be explored, and copper-free click DNA ligation could be carried out in a cellular context. The future of click nucleic acid ligation holds great promise, and thoughtful and imaginative application of this technology could lead to major advances.

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BIOPGRAPHICAL INFORMATION

Afaf H. El-Sagheer studied Chemistry and was appointed as a demonstrator at Suez Canal University (Egypt). She completed her Ph.D. in Chemistry at Southampton University in 2000 then returned to Egypt to take up a lectureship at Suez Canal University. She is now Associate Professor, and is currently on a sabbatical leave working with Tom Brown on applications of oligonucleotide chemistry to biology and nanotechnology.

Tom Brown was appointed to a lectureship at Edinburgh University in 1985 where he became Professor of Nucleic Acid Chemistry. He moved to Southampton University in 1995 where he is currently Chair of Chemical Biology. His research interests center on Nucleic acids and the application of oligonucleotide chemistry to biology and medicine. He has received numerous awards including the recent Royal Society of Chemistry prizes for Nucleic Acid Chemistry and Interdisciplinary Research. He is co-founder of three Biotech companies.

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